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THE EFFECT OF TREATING THE VIRUS OF TOBACCO MOSAIC WITH THE JUICES OF VARIOUS PLANTS

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During the period that the mosaic disease of tobacco has been under investigation in this laboratory, many attempts have been made to transfer the disease, through cross inoculation, to the pokeweed, *Phytolacca decandra*. Five operators have at different times endeavored to make the transfers referred to, each employing a somewhat different technique. In no instances have results been obtained that might be interpreted as a positive indication of the susceptibility of the pokeweed to the tobacco form of mosaic.

Although at that time no experiment had been made here in the direction of testing the possibility of transfer through insect agency, it seemed quite probable that the pokeweed might be immune. It occurred to the junior author that it might be of interest to determine the effect of the juice of the pokeweed on the tobacco virus. Accordingly, a preliminary experiment was arranged, and it was determined to include in this the effect upon the tobacco mosaic agency of both the juice from healthy pokeweed and that from pokeweed affected with the mosaic peculiar to that host.

Juice or crude sap was then prepared from (1) healthy pokeweed, (2) pokeweed affected with mosaic, and (3) tobacco plants

affected with tobacco mosaic. In each case the procedure was as follows: The younger shoots and leaves were ground up in a meat or food grinder and the resulting semi-fluid mass was thrown into a muslin bag and the juice pressed out. The juice was then filtered through absorbent cotton. The undiluted filtered juice was then used as indicated later. Adequate precautions were taken as to the cleanliness of all vessels involved. The meat grinder and other articles required were, after preliminary cleaning, soaked several hours in hot water containing ½ per cent formaldehyde, and afterwards boiled in plain water. In these and in later experiments where pipettes and other glassware were required, such vessels were invariably dry-sterilized.

In the preliminary experiment, 1 part of the tobacco juice from diseased plants was mixed with 99 parts of each of the 2 types of pokeweed juice, and as a partial control on the dilution and the infectivity of the virus, 1 part of the same diseased tobacco juice was added to 99 parts of sterile distilled water. Further control was not used at the time owing to a scarcity of suitable plants for the inoculation work. The fluids as diluted for inoculation were permitted to stand 3–4 hours prior to their use. The technique of inoculation is described later. Twenty healthy young tobacco plants were inoculated with each of the 3 lots of material, 4 cc. of inoculum being used for each lot. The inoculations were made June 12, and all of the 20 control plants (diseased juice 1 part, water 99 parts) were diseased by June 23, while none of the 40 plants inoculated with the diseased tobacco juice made up in pokeweed juice showed the least indication of mosaic on June 30, when the experiment was closed. These results were so definite that it was determined to repeat the work and to use a variety of plant juices when opportunity offered.

The method of inoculation employed in the preliminary experiments and in that subsequently reported is one that has been adopted as a standard in this laboratory and it requires a brief description. It is preferred to employ young plants with stems about 2 inches high. When possible, the plants to be inoculated are arranged in place about 1 week in advance so that sporadic occurrences of the disease may be promptly detected. Three inoculations are made in each plant, 1 on the stem near the ground,

another farther up and in a leaf axil—so as to reach the young bud—and the other in the immediate region of the terminal bud. The quantity of inoculum required is about 2 cc. for each 10 plants. A large drop of the inoculum may be placed with a glass rod on the spot desired, and with a small inoculating needle many pricks are made through this, thus working the fluid into the tissue. Before passing to the next plant the needle is flamed, then dipped in alcohol, and burned off. The usual precaution is taken with respect to touching plants, or if plants must be touched, the hands are cleansed between operations. Because no disease has appeared in any lot of plants, it is not safe to assume freedom from disease, so that every plant in any lot is treated as though it might convey disease. Inoculated plants are kept under observation for 28–30 days in all cases where more than preliminary data are required.

Preparing the crude sap as already described, experiments were arranged with shoots and leaves of pokeweed, Jimson weed (*Datura Stramonium*), geranium (*Pelargonium* sp.), cotton, and squash, with Irish potato tubers, sweet-potatoes, and apples of the variety Ben Davis. In each case the juice from diseased tobacco plants was diluted, as shown in table I, with the juice from the plant the influence of which was to be tested. In these experiments, the mixed juices were allowed to remain at room temperature about 2 hours, and then placed in a refrigerator, at 3° C., until used the following morning (15–18 hrs.). The undiluted juices used as control were similarly exposed.

From the table it is again clear that pokeweed juice effectively inactivates the agency of tobacco mosaic in a relatively short time. Even when the virus is diluted only 5 times with the pokeweed juice the inhibition is complete. Inactivation is shown by the juice of Jimson weed when the latter is in relatively high concentration, and the juice of the geranium is also to some extent effective. On the other hand, cotton, squash, potato, sweet-potato, and apple exert no injurious influence at the concentrations tested.

It became of much interest to determine if the influence of pokeweed juice especially might result from some relatively simple chemical factor, or if it might be far more complex, possibly

analogous to agglutination. The possibility that the reaction of the juice might be an important factor suggested itself, but colorimetric tests made it clear that the H-ion concentration was

TABLE I

EFFECT OF VARIOUS PLANT JUICES ON THE PATHOGENICITY OF THE TOBACCO MOSAIC VIRUS. TEN PLANTS WERE INOCULATED IN EACH CASE

Juices constituting inoculum	Dilution	Number diseased
Diseased tobacco, dist. water	1 : 10	All (in 15 days)
Diseased tobacco, dist. water	1 : 100	All (in 11 days)
Diseased tobacco, pokeweed	1 : 5	None
Diseased tobacco, pokeweed	1 : 10	None
Diseased tobacco, pokeweed	1 : 25	None
Diseased tobacco, pokeweed	1 : 50	None
Diseased tobacco, pokeweed	1 : 75	None
Diseased tobacco, pokeweed	1 : 100	None
Pokeweed	None	1 (after 4 wks.) (apparently accidental)
Diseased tobacco, Jimson	1 : 10	All (in 9 days)
Diseased tobacco, Jimson	1 : 100	None
Jimson	None	None
Diseased tobacco, Irish potato	1 : 10	All (in 9 days)
Diseased tobacco, Irish potato	1 : 100	All (in 9 days)
Irish potato	None	None
Diseased tobacco, sweet-potato	1 : 10	All (in 11 days)
Diseased tobacco, sweet-potato	1 : 100	9
Sweet potato	None	None
Diseased tobacco, geranium	1 : 10	8 (in 15 days)
Diseased tobacco, geranium	1 : 100	3
Geranium	None	None
Diseased tobacco, apple	1 : 10	All (in 14 days)
Diseased tobacco, apple	1 : 100	8 (in 15 days)
Apple	None	None
Diseased tobacco, tobacco	1 : 10	All (in 12 days)
Diseased tobacco, tobacco	1 : 100	All (in 12 days)
Healthy tobacco	None	None
Diseased tobacco, cotton	1 : 10	All (in 13 days)
Diseased tobacco, cotton	1 : 100	All (in 13 days)
Diseased tobacco, squash	1 : 10	All (in 9 days)
Diseased tobacco, squash	1 : 100	All (in 9 days)
Squash	None	None

higher in the apple juice than in any other tested, and this last exerted no injurious influence.[1] Moreover, experiments then in progress, to be reported elsewhere, indicated a relatively high tolerance of the mosaic virus towards acids. In view of the following facts, namely, that pokeweed is a host for a form of mosaic, that Jimson weed is credited with several forms of mosaic, and

[1] Employing electrometric methods, a subsequent test made by Mr. E. R. Ranker and Miss Fanny Fern Smith gave a P_H of approximately 6.0 for the juice of the pokeweed used.

that even geranium exhibits a disease tentatively classed as a mosaic, the influence of the juices of these plants must be regarded as specific with reference to the tobacco mosaic agency. However, in order to determine if pokeweed juice possesses general germicidal properties, cultures in small Erlenmeyer flasks were arranged with sterile and unsterile (natural) juice, and both lots were inoculated with *Aspergillus niger*. The fungus grew promptly and profusely on both lots, indicating no general inhibiting effect.

The toxicity of pokeweed juice was further tested, using as an indicator the growth of *Bacterium prodigiosum* Lehm. & Neum. In carrying out the experiments with this organism a strong bacterial emulsion was prepared from a fresh nutrient agar slant culture. This emulsion was then added to pokeweed juice,[1] or to an equal quantity of distilled water, as control. These mixtures were allowed to stand for 2 hours, after which suitable dilutions were prepared and plates were poured. Every possible care was taken with the samples employed in the preparation of the dilution cultures to take the sample from the vessel in such manner that the pipette did not come in contact with the walls of the vessel where organisms might occur which had not had free access to the fluid used. In the first series of cultures the original bacterial emulsion was diluted (1) 1:5 with pokeweed juice; (2) 1:25 with pokeweed juice; and (3), for control, 1:25 with distilled water. The results are given in detail in table II.

The result of the foregoing test is convincing proof that no toxicity of pokeweed juice is exhibited toward this species of bacteria. In explanation of the table it should be pointed out that the data with the 2 dilutions of pokeweed juice are closely comparable, while the result from dilution with distilled water shows apparently a very much smaller number of bacteria present. This, however, was anticipated and is in harmony with results obtained from a preliminary series not here reported in detail. This preliminary series brought out the fact that, on

[1] The pokeweed juice used in these experiments was prepared as previously described except that in this case, in order to minimize contaminations, the pokeweed leaves and shoots were first treated for 4 hours with 20 per cent Javel water, after which they were washed in sterile distilled water.

TABLE II

EFFECT OF POKEWEED JUICE ON BACTERIUM PRODIGIOSUM; EXPOSURE TO JUICE 2 HOURS

Treatment	Plate dilution	No. colonies, 4 days	Average, colonies per cc.
Bacterial emulsion diluted 1 : 5 with pokeweed juice	1/10	Numerous	51,930
	1/100	768	
	1/1000	39	
	1/10000	4	
Bacterial emulsion diluted 1 :25 with pokeweed juice	1/10	960	10,800
	1/100	108	
	1/1000	12	
Bacterial emulsion diluted 1 : 25 with distilled water	1/10	71	1,055
	1/100	14	
	1/1000	0	

standing, an emulsion of this bacterium in distilled water shows an apparent decrease in the number of organisms. It is not the purpose of this paper to determine the cause of this decrease though it may be a simple aggregation phenomenon. For verification of this observation another experiment was carried out, the results of which are briefly given in table III. In this experiment the bacterial emulsion was diluted, as will be seen, with distilled water and with pokeweed juice to the same extent. Plates were poured immediately after the bacterial emulsions were diluted, and then after intervals of 1 and 2 hours respectively.

TABLE III

BACTERIUM PRODIGIOSUM IN DISTILLED WATER AND IN POKEWEED JUICE

Treatment	No. colonies, initial	No. colonies, after exposure of 1 hour	No. colonies, after exposure of 2 hours
Bacterial emulsion diluted 1 : 25, distilled water	20,300	——	7,050
Bacterial emulsion diluted 1 : 25, pokeweed juice	29,850	44,050	42,500

No discussion of table III is required further than to point out again the diminution, on standing, of the number of colonies in the case of the emulsion diluted with distilled water. The figures for the emulsion diluted with pokeweed juice are sufficiently comparable when it is recalled that this is not filtered juice, and

accordingly represents a minute suspension, in itself a sufficient cause for some variation in numbers.

These experiments were further suggestive of a specific inactivating effect of the pokeweed juice towards the agency of mosaic disease. The cases of geranium and of Jimson weed have not been more closely analyzed.

It seemed desirable to determine the possible relation of the larger colloidal particles in the pokeweed juice to inactivation, but up to the present only a few preliminary experiments have been made. These, however, are not without suggestion. Pokeweed juice was filtered through a cylindrical, porcelain atmometer cup under a pressure of one-half atmosphere, and the filtrate thus obtained was used in treating the mosaic virus subsequently used in inoculations, as in table I. When the relation of virus to filtrate was 1 : 10, the incidence of infection was 9 out of 10 plants inoculated; when the relation was 1 : 100, the virus was completely inactivated. Filtration through such a filter seems therefore to reduce the effect of the juice. It may be stated that the filter employed permits the mosaic virus to pass through but does not permit *Bacterium prodigiosum*.

Some of the same lot of pokeweed juice was diluted with an equal quantity of distilled water and then centrifuged for 30 minutes at 1500 revolutions per minute. The effect of the supernatant liquid on the virus was determined, as before, by inoculating 10 tobacco plants. With the relation of the diseased juice to this diluted liquid 1:10, 2 plants developed the disease, again indicating some loss of inactivation capacity.

It seemed conceivable that inactivation of the mosaic virus might be due to adsorption. It was not possible to determine this merely by increasing relatively the quantity of diseased tobacco juice, since this dilutes the pokeweed juice. Concentration of the diseased tobacco juice by evaporation would also be subject to criticism from another angle. Further work on the chemical or physical nature of this inactivation is planned.

For the moment the data in the 3 experiments given in table IV show further the influence of the ratio of diseased juice to pokeweed juice as developed through infection experiments, and thus supplement table I. The experiments were made under con-

ditions similar to those reported in table 1, but at a different time. The progressive increase in the incidence of disease with the increasing amount of virus locates rather definitely, in conjunction with the data of table 1, the inactivation capacity of the pokeweed juice.

TABLE IV

Juices constituting inoculum	Dilution	Result
Diseased tobacco, pokeweed	1 : 1	2 diseased (in 8 days)
Diseased tobacco, pokeweed	5 : 1	8 diseased (in 14 days)
Diseased tobacco, pokeweed	10 : 1	9 diseased (in 9 days)

Upon the completion of the experiments with pokeweed juice reported in table 1, the results were regarded, and apparently properly so, as a simple case of inactivation. Nevertheless, it was considered desirable to ascertain if in the plants used in experiments 3–8 inclusive there might exist such a mild form of the disease as to be essentially without external symptoms. Accordingly, leaves of these plants were used directly in the inoculation of fresh young tobacco plants, but without visible result. Likewise, one-half (5) of each lot of plants used in experiments 3–8, table 1, were inoculated with diseased tobacco juice, untreated, and the entire lot of 30 plants exhibited the characteristic mosaic symptoms in less than 15 days, indicating, as anticipated, not the least suggestion of a modification of susceptibility to mosaic.

The specific nature of this inactivation of the tobacco mosaic virus, especially by pokeweed juice, has seemed of sufficient importance to justify the projection of further experiments in the direction of ascertaining the physical and chemical properties of this juice.

DETERMINATION OF TOTAL NITROGEN IN PLANTS AND PLANT SOLUTIONS: A COMPARISON OF METHODS WITH MODIFICATIONS

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The analyst who has had considerable experience in the determination of nitrogen is impressed by the fact that some substances are analyzed with more ease and accuracy than other substances. Many factors are involved, not the least of which is the combination of the various forms of nitrogen in the sample to be analyzed. For example, if we consider the determination of total nitrogen in whole plants (peas, wheat, barley, etc.) including the residual nutrient solutions in which they grew, amino-, amide-, and some ammonia-nitrogen are present in the plants in addition to nitrate- and possibly ammonia-nitrogen in the residual solutions. In such a case the determination of total nitrogen presents problems that challenge the accuracy of the various methods used.

Two methods are used generally: (1) some modification of the Devarda method, and (2) the official salicylic-thiosulphate method (Assoc. Off. Agr. Chemists, '21, p. 8; I, 28). The latter method has been criticized severely by several investigators whose data have been interpreted as indicating the limitations and defects of any method based on the reduction of nitrates in acid medium. Further reference will be made to these criticisms after the presentation of data. On the other hand, the Devarda method for total nitrogen in the presence of organic matter is time-consuming, since (1) there is a preliminary alkaline distillation with the alloy to collect the ammonia obtained from the reduction of the nitrates and other substances acted upon in the process (Allen, '15; Davisson, '18), and (2) the organic matter and remaining nitrogen must be subjected to a Kjeldahl digestion followed by a second distillation into the same or a second lot of standard acid. Unfortunately, then, neither of these methods is entirely satisfactory for the determination of total nitrogen in plants and plant solutions when the two are present in the same sample.

In a study of nitrogen fixation by higher plants the total nitrogen content of the plants and residual nutrient solutions was

determined. The official salicylic-thiosulphate method was used. Extremely inaccurate results were obtained. From nutrient solutions containing respectively 400, 300, 200, 100, 50, 25, and 10 mgs. of nitrogen per 950 cc., only 68, 67, 30, 62, 62, 54, and 27 per cent of the nitrogen was recovered. From solutions containing smaller quantities of nitrogen than 10 mgs. the amounts recovered were usually less than for the blank, that is, there was a loss of nitrogen from the reagents used. A total of about 60 determinations was made, and there was no agreement among the results obtained. The greatest losses of nitrogen occurred from those samples containing whole plants in addition to the residual nutrient solutions. In several cases there was a visible evolution of nitrogen dioxide fumes, and duplicate determinations varied widely. These data tend to corroborate the statements of those investigators who have criticized the official salicylic-thiosulphate method.

In spite of these criticisms, however, it was thought advisable to attempt some modifications that might overcome the difficulties encountered. Several possibilities were tried, and from the results of these tests it was tentatively determined that the inaccuracy of the method was due, primarily, to the presence of water at some stage during the process of acid digestion. Certain details of manipulation seemed to influence the determination to a limited degree.

A modification of the official method was devised, and its accuracy for the determination of total nitrogen was tested out on the various forms of nitrogen. For purposes of comparison, simultaneous determinations on samples from the same stock, measured by the same pipettes and at the same temperature, were made by a modification of the Devarda method. The procedure for this comparison method was as follows: (The procedure for the modified official method will be given later.)

I. *If organic matter is not present.*—Place the sample in an 800-cc. Kjeldahl flask; add 1.0 gm. of Devarda alloy for every 60–70 mgs. of nitrate-nitrogen present[1]; add a small piece of paraffin; make up to

[1] Subsequent to this investigation the work of Burrell and Phillips ('25) has been published. These investigators used 1 gm. of alloy; they were dealing with small amounts of nitrogen and evidently did not determine the limits of their method. The reduction limits of 1 gm. of alloy is approximately 70 mgs. of nitrogen in the form of nitrate-nitrogen.

a total volume of 150 cc.; add 6 cc. of 10 per cent sodium hydroxide; connect to the distillation apparatus and distill into standard acid at slow boiling for 1 hour. Titrate the standard acid to neutrality.

II. *If organic matter is present.*—Proceed as in I; continue distillation to as low a volume as is safe; disconnect. Add 45 cc. of concentrated suphuric acid and 10 gms. of anhydrous sodium sulphate; digest for 1 hour after the copper color appears (the mixture remains milky due to aluminum and zinc precipitates); cool; make up to an estimated volume of 400 cc. and distill as for the modified official method.

All the determinations reported were made of the following forms of nitrogen:

1. Nitrate-nitrogen as $NaNO_3$ which was twice recrystallized from Merck's blue-label grade. The solution used was such that 1 cc. was equivalent to 1 mg. of nitrogen.

2. Ammonia-nitrogen as $(NH_4)_2SO_4$ which was recrystallized from Merck's blue-label grade. The solution used was such that 1 cc. was equivalent to 1 mg. of nitrogen.

3. Amino-nitrogen as glycine, an Eastman Kodak Company product. The solution used was such that 1 cc. was equivalent to 0.91 mg. of nitrogen.

4. Amide-nitrogen and amino-nitrogen as asparagine, a Merck product. The solution used was such that 1 cc. was equivalent to 0.95 mg. of nitrogen.

5. Total organic plant nitrogen as found in 6-day-old wheat seedlings; 10 such seedlings produced 7 mgs. of nitrogen with but little variation from this average. It would have been somewhat more accurate had smaller seeds been used, in which case the larger number used would have appreciably decreased the variation from the average.

The acid and alkali used in titration were standardized against benzoic acid obtained from the U. S. Bureau of Standards (sample No. 39B). A total of 190 determinations, including the preliminaries, was made. The results obtained were subjected to a statistical analysis, the data of which, expressed as average percentages, are given in table 1. The probable error of the arithmetical mean was calculated by the formula

$$\left(E_m = \frac{0.6745\sigma}{\sqrt{n}}\right).$$

TABLE I

RECOVERY OF TOTAL NITROGEN

(average percentages and probable error)

Sample determined	Recovery by Devarda method		Recovery by modified method		No. of trials
	Sample first evptd. to dryns.	Sample plus water	Sample first evptd. to dryns.	Sample plus water	
50 mgs. nitrate-N.	97.7 ±.19	98.4 ±.03	100.4 ±.06	62.2 ±.38	8
100 mgs. nitrate-N.	68.5*	72.4* 99.9‡ ±.24	99.2 ±.10	17.8 ±.81	13 3
50 mgs. nitrate-N. 10 mgs. ammonia-N.	98.5 ±.23	99.4 ±.22	98.0 ±.06	36.7 ±1.80	12
50 mgs. nitrate-N. 10 mgs. amino-N.	97.7 ±.14	99.4 ±.04	99.3 ±.14	51.7 ±4.03	12
50 mgs. nitrate-N. 10 mgs. {amino-N. amide-N.	97.5 ±.09	96.3 ±.10	99.3 ±.28	47.6 ±.29	15
50 mgs. nitrate-N. 7 mgs. plant-N.†	99.6 ±.09	98.8 ±.09	100.2 ±.32	64.6 ±.23	12
50 mgs. nitrate-N. 7 mgs. plant-N.† 0.5 cc. H_2SO_4	These samples were not determined, as during evaporation heavy NO_2 fumes were given off.				
50 mgs. nitrate-N. 7 mgs. plant-N.†‖	100.3 ±.56		99.7 ±.69		14
50 mgs. nitrate-N. 7 mgs. plant-N.† 1 cc. N/10 NaOH	99.2 ±.52		99.1 ±.62		6
50 mgs. nitrate-N. 0.5 gm. sucrose	98.1 ±.64	99.5 ±.00	92.7 ±.90	33.2	10

* 1.0 gm. of Devarda alloy used.

† Supplied as the nitrogen content of ten 6-day-old wheat seedlings.

‡ 2.0 gms. of Devarda alloy used.

‖ This solution was adjusted to neutrality prior to determination.

From a study of the data of table I certain facts are evident:

1. The presence of free water in the sample is the determining factor for the accuracy of a method dependent upon the reduction of nitrates in acid medium (the minimum amounts of water that may be present and not decrease the accuracy of such a method

have not been determined—it is recommended, however, that no more than just a trace of free water be present).

2. When the sample is practically dry the official method as here modified is somewhat more accurate for the determination of total nitrogen than the Devarda method used.

3. The modified method is accurate for the determination of amino-, amide-, ammonia-, nitrate-, and total plant-nitrogen and combinations of these forms of nitrogen in plants and plant solutions.

4. If sugar is present in abundance a slight loss of nitrate-nitrogen may occur, due to the reducing action of the sugar. This loss would be very slight in actual practice since the nitrate-nitrogen content of plants is small.

The procedure for the modified official method as used in this investigation is as follows:

Place the sample in an 800-cc. Kjeldahl flask; adjust to neutrality or make slightly alkaline; if water is present evaporate *just to* dryness on a water bath under vacuum. Add 35–40 cc. of salicylic acid mixture (1.0 gm. of salicylic acid to 30 cc. of concentrated nitrogen-free sulphuric acid); mix thoroughly and allow to stand for at least an hour with occasional shaking (if organic matter is present, stopper tightly with a rubber cork and allow to stand over night). Add 5 gms. of sodium thiosulphate and heat for 5 minutes with a low flame; cool; add 7–10 gms. of anhydrous sodium sulphate and a pinch of copper sulphate. Digest for an hour at the boiling point after the solution clears; just before the solution solidifies dilute to an estimated volume of 400 cc.; cool completely. Add a small piece of paraffin, 100 cc. of a saturated solution of sodium hydroxide, and a piece of mossy zinc; connect immediately to the distillation apparatus and distill 150–200 cc. over into standard acid during a period of 1 hour. Titrate the standard acid to neutrality with standard alkali and calculate the amount of nitrogen present.

Subsequent to this investigation 380 determinations were made by the above method with an accuracy equivalent to that here reported. Some of the samples determined had a total nitrogen content as high as 400 mgs., in which case the amount of salicylic acid mixture was increased to 45 cc. Some of the samples determined had a total nitrogen content of 2 mgs. per 950 cc. of solution; the solutions (950 cc. in the sample) were evaporated just to dryness on a water bath under vacuum and an accurate recovery was obtained. Most of the determinations

were made on samples composed of whole plants of wheat, barley, or peas, plus the residual nutrient solutions in which they grew. If this modified method be in error all attempts to locate that error have failed and any suggestions or criticisms are welcome.

Attention to detail is essential to accurate determinations; for this reason it is well to mention a few details of manipulation that have been found of value:

1. It is the habit of some analysts to wash down the neck of the Kjeldahl flask during the process of digestion; in the interests of personal safety and certainty of results this should be avoided. Such a practice introduces water into the sample and operates against the advantages of a previous evaporation under vacuum. If sulphur collects in the neck of the flask it can be removed easily by heating gently and uniformly the neck of the flask in the open flame; by using a strong hot flame during the last hour of digestion the accumulation of sulphur is almost entirely avoided.

2. If excess solid organic matter is present in the sample it may be necessary to increase the amount of salicylic acid mixture used in order to maintain a liquid condition of the contents during the first part of digestion.

3. The open flame should be allowed to come in contact with only that portion of the flask which is covered by the solution. Use an asbestos ring to prevent this (Paul and Berry, '21).

4. In the presence of organic matter, the tendency of the digesting mixture to foam and spew out presents an irritating problem. This loss by foaming has been entirely overcome in this laboratory by allowing the acid to thoroughly disintegrate the solid portions of organic matter, without heat, over an approximate 12-hour period It is advisable to redistribute the acid by occasional shaking. It is necessary to stopper the flasks tightly with rubber corks to prevent the absorption of ammonia fumes. The practice used by the author has been to add the acid and allow the mixture to stand over night. Of the 380 determinations mentioned above, not one has been lost due to foaming when this method was followed.

5. For evaporation of the samples a 5-hole, constant-level, gas-heated water bath is used; the flasks are held on this bath in an

inclined position by 2 notched wooden supports and connected to an ordinary water filter-pump by a series of 5 corks (rubber) and 4 Y-tubes. A 3-liter safety bottle is placed between the filter-pump and the flasks on the water bath to prevent the entrance of water into the flasks when, for any reason, the water pressure becomes reduced. In operation, a partial vacuum is quickly developed and ebullition proceeds at a rapid rate, providing all rubber connections are sufficiently thick-walled to withstand the vacuum developed. Evaporate the sample just to dryness, not to an ash-dry condition. Release the vacuum slowly before removing the flasks; if released rapidly the flasks will crack.

6. A few Pyrex glass beads or small angular pieces of broken Pyrex glass placed in the flask will facilitate evaporation and subsequent digestion; they may be used over and over again.

7. "Bumping" during distillation is a question of concentration and relative abundance of insoluble substances present—at a dilution of 400 cc. practically no "bumping" was experienced.

8. To determine the correct amount of alkali to use in distillation the following test is of value: When ready to distill, add 2 drops of phenolphthalein indicator; add the paraffin, sodium hydroxide, and zinc; after the flask is connected to the distillation apparatus and the flame is adjusted, shake the flask vigorously and if the correct amount of alkali has been added the pink color of the indicator will flash through the solution for 1 to 2 seconds and disappear. If the pink color lasts for more than 2 seconds it is advisable to add more alkali; if the color disappears in less than 1 second a useless excess of alkali is present. Once adjusted the amount of alkali remains practically constant as long as the amount of acid used in digestion is not varied.

In the first part of this paper reference was made to certain criticisms against various methods which are based on the reduction of nitrates in acid medium, and it was also shown that those criticisms against the official method (Assoc. Off. Agr. Chemists, '21) were satisfactorily confirmed in this laboratory. These same criticisms are not applicable, however, to the modified method here reported. A critical study of the data upon which these criticisms are based reveals the fact that the moisture content of the samples was not adequately controlled.

It is pertinent to this investigation, therefore, although no attempt has been made to go into the literature, that all such criticisms which did come to notice[1] be considered. In doing so it is to be remembered: (1) that the accuracy of the modified official method here proposed has been tested out for plants and nutrient solutions only, (2) that, while no difficulty is anticipated in using this method on other biological substances in watery medium (for example, soil extracts), no broad generalizations are advanced until these tests are actually made, (3) that a criticism of the principle upon which this method is based, that is, the acid reduction of nitrates, is also an indirect criticism of the method here used, though the particular method under criticism may be that of Jodlbauer (Metge, '18, p. 30), Förster (Krische, '06, p. 71), etc.

After the completion of this investigation a reference to the work of Allen ('15) became available. Allen's work was very carefully done and is referred to often. In his summary, referring to reduction methods, he concludes that "of such procedures only the modified Devarda and aluminium reduction methods gave promise of meeting our requirements." The data presented indicate that these methods (that is, 2 Devarda methods and 1 aluminium reduction method) were the only ones actually tested out by Allen ('15). He rejected the acid methods of nitrate reduction, apparently upon the evidence presented by Mitscherlich and Herz ('09), as follows:

"Mitscherlich and Herz conducted an extended investigation on the perfection of an accurate Kjeldahl method which would include all forms of nitrogen, in course of which they studied, among other sources of error, the question of the reduction of nitric nitrogen. Using phenolsulfonic acid and zinc dust, sodium hydroxide, and zinc-iron dust, Jodlbauer's method, and Förster's method, they were unable to obtain the theoretical amount of ammonia from nitrate." (Allen, '15, p. 522).

Reference was then made to the work of Mitscherlich and Herz ('09), whose data is an excellent tribute to accurate procedure.

[1] After this paper had been sent to the publishers a reference to the work of W. E. Loomis (Am. Soc. Hort. Sci. Proc. 1924: 365–370) was found. From the data of 7 reported determinations the official salicylic-thiosulphate method is discarded by Loomis. The criticisms advanced against this method and against the principle of the acid reduction of nitrate are essentially the same as the criticisms previously advanced by Gallagher ('23), which will be considered later in this paper.

For the purposes of this study, however, attention must be called to certain facts pertaining to their report ('09):

1. Of the 40 pages devoted to the report, only 12 (pages 307–318) are concerned with a study of any method based on the reduction of nitrates in acid medium.

2. Through their data they give no detailed statement of procedure for any of the many methods they used, except for that modification of the Devarda method which they recommend (Mitscherlich and Herz, '09, p. 280). They refer to the other methods by name only (for example, Förster, Jodlbauer, etc.), and it is logical to assume, therefore, that the procedures for the methods used were those in common use at the time. For example, the Förster method in use at that time was in part as follows:

"0.5 g Salpeter (50 ccm der Lösung 10:1000) werden in einem Kjeldahlkolben mit 15 ccm einer 6%igen Phenolschwefelsäure oder mit 15 ccm einer 6%igen Salizylsäure-Schwefelsäure vermischt, . . ." etc. (Kirsche, '06, pp. 71–72).

The procedure for the Jodlbauer method is very similar:

". . . : 250 ccm Wasser werden mit 25 ccm Phenolschwefelsäure im Kjeldahl-Kolben versetzt und nach Zugabe einiger Sandkörnchen möglichst weit eingedampft. . . ." etc. (Metge, '18, p. 20, no. 13).

Attention is called to the presence of water in the samples determined by these methods.

3. Finally, attention must be called to the fact that the determinations upon which Mitscherlich and Herz ('09) based their conclusions and to which Allen ('15) referred were made upon samples in water solutions or upon soil extracts, some of which were very dilute. Most of the tables presented have headings similar to the following examples:

"Analyse einer Kaliumnitratlösung mittels der Zink-Eisenstaub-Reduktionsmethode" [table 37, p. 316].

"Versuche zur quantitativen Reduktion des Salpeterstickstoffs einer Kaliumnitrat-Lösung" [table 38, p. 317].

"Stickstoffbestimmung in einem Bodenextrakt nach der Zinkeisenstaub-Reduktionsmethode" [table 36, p. 315].

In explanation of the data in table 34, p. 313, the following occurs:

"Nach der Reduktion der auf je 1 L verdünnten Mengen der Kaliumsalpeterlösung durch zwei Stunden langes Erhitzen auf fast 100° unter Zusatz von 5 g Zinkstaub und 5 ccm Phenolschwefelsäure, nach darauffolgendem Eindampfen und Destillieren wurden folgende

Ammoniakmengen entsprechend ccm N/50 H_2SO_4 im Destillat festgestellt:" [table 34, page 313].

In a few cases the solutions apparently were evaporated and theoretical recovery of nitrogen was not obtained (Mitscherlich and Herz, '09, p. 316). In most of these cases some acid was added (table 32, p. 312; table 35, p. 314) prior to evaporation and in the other cases no statement can be made due to the absence of specific details of procedures.

From a study on the determination of nitrites and nitrates in plants Strowd ('20) concluded that "the determination of nitrates in plants by finding the difference between the Kjeldahl-Gunning-Arnold method and the Kjeldahl method modified to include nitrates is unsatisfactory." Strowd attempts to explain this discrepancy on the basis that "appreciable amounts of nitrate were apparently reduced without zinc and salicylic acid." These deductions were based on 2 determinations only (Strowd, '20, table 1); one being a determination of the nitrate-nitrogen content of a "Pure $NaNO_3$ solution," and the other a "Pure $NaNO_3$ solution + nitrate-free plant extract." By calling attention to these facts it is not implied that the difference between the nitrogen obtained by the Kjeldahl-Gunning-Arnold method and the modified official method, as here reported, would be accurate for the determination of nitrate-nitrogen only—this is another problem. The purposes of this investigation are served by calling attention to the presence of water in the samples determined (Strowd, '20).

For the determination of total nitrogen in plants Gallagher ('23, p. 67) concludes that "nitrate reductions are invariably essential in dealing with Kjeldahl estimations of plant products, since the plant contains nitrates at nearly all stages of growth." He recommends a Devarda method to accomplish this and discards the principle of acid reduction of nitrates on a purely theoretical basis as follows:

"In the estimation of nitrates by means of acid reducing agent a serious objection exists when amino groups, whether in the form of amino acids, or of proteins, etc., be present. The first step in the reduction of nitrates would appear to be the formation of nitrites. In the presence of acid, nitrous acid is formed, which, of course, will quickly react with the amino groups of amino acids, etc., with liberation of nitrogen."

Under certain conditions, and for the method (Ulsch) involved this may be a true statement of fact, but it is not true as a generalized criticism of the reduction of nitrates in acid medium. In acid medium, for example, concentrated sulphuric acid, in the absence of water, and in the presence of either phenol or salicylic acid, the above generalization probably does not hold true. Skeleton equations to illustrate the transformation of the nitric-acid radicle into ammonia may be somewhat as follows, in the case of phenol:

(1) $C_6H_5.OH + HNO_3 = C_6H_4.OH.NO_2 + H_2O$

(2) $C_6H_4.OH.NO_2 + 3H_2 = C_6H_4.OH.NH_2 + 2H_2O$

(3) $C_6H_4.OH.NH_2 + NaOH = C_6H_4.OH.ONa + NH_3$

A very similar transformation takes place with salicylic acid; the reduction process is hastened by the addition of zinc or sodium thiosulphate. By equation (1) it is evident that nitric acid is responsible for the nitrification of the phenol- or salicylic-acid molecule. The nitric acid is obtained from the action of the sulphuric acid on the nitrate present:

(4) $NaNO_3 + H_2SO_4 = NaHSO_4 + HNO_3$

If water is present in the sample to be determined it is probable that the nitric acid will be diluted below the point at which it can quantitatively nitrify the phenol or salicylic-acid molecule; if so, the instability of the nitric-acid molecule will be manifest and there will be a loss of nitrogen-dioxide gas:

(5) $4HNO_3 = 2H_2O + 4NO_2 + O_2$

In fact, in many cases when even small amounts of water are present, visible amounts of nitrogen-dioxide fumes may be given off. The above generalization by Gallagher ('23, p. 64) is based on the reported data of 2 determinations only; the first one being the analysis of ". . . . a *solution*[1] containing nitrate only, . . . ," and the second being the analysis of ". . . a *solution* containing 14.84 mg. of nitrate nitrogen and 9.13 mg. of nitrogen in the form of asparagine. . . ." He obtained a nitrogen loss of approximately 20 per cent.

[1] Italics ours.

SUMMARY

For the determination of total nitrogen in plants and plant solutions the official salicylic-thiosulphate method has proved inadequate. The inaccuracies of this method are demonstrated to be due, primarily, to the presence of water in the sample under analysis. A modification of the official salicylic-thiosulphate method is proposed and certain details of manipulation are discussed. Under the conditions of this investigation this proposed modified method has proved to be approximately twice as rapid and just as accurate as a modified Devarda method used for comparison purposes. Some of the criticisms advanced against the principle of acid reduction of nitrates are reviewed.

It is with pleasure that the author acknowledges his indebtedness to Dr. B. M. Duggar, whose kindly interest, many suggestions, and keen criticisms have been a constant source of strength in this investigation. Acknowledgements also are made to Dr. George T. Moore for the use of the facilities of the Missouri Botanical Garden, and to Dr. E. S. West, Dr. Paul S. Rider, and Dr. Roland La Garde for the timely suggestions and assistance they have gladly given.

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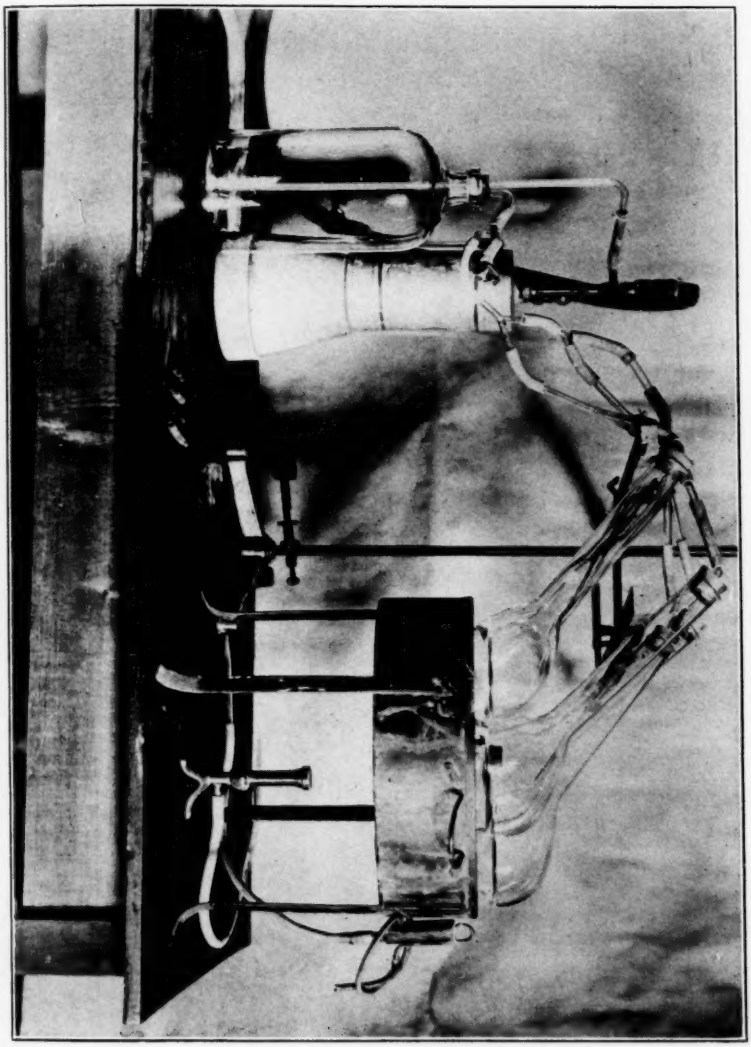
Explanation of Plate

PLATE 19

Apparatus used for the evaporation of the samples under vacuum.



RANKER—DETERMINATION OF TOTAL NITROGEN

COLLOIDAL SULPHUR: PREPARATION AND TOXICITY

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There is urgent need for a fungicide which may be used effectively, without danger of injury, on a variety of plants under diverse conditions.

Sulphur has long been known to have fungicidal properties. Previous to 1880 it was almost the only fungicide in use, doubtless due, in large part, to its abundance and low cost. In the years 1848, 1852, and 1853, during the serious outbreaks of the grapevine mildew in France (Bourcart, '13) it played an important part as a fungicide. The diversity of the forms and compounds of sulphur has had an important bearing upon the use of this substance as a fungicide. The application of a mixture of lime and sulphur for the control of grape mildew was recorded as early as 1833 (Scott, '08), before the use of fungicides became general. Lime sulphur is at present the form of sulphur most widely employed and it ranks high among the standard spray mixtures. However, the use of this spray has been limited to a certain extent because of the caustic effect upon the foliage of certain plants, due to the soluble sulphide content. Serious burning is often reported from its use on apples, which are among those plants least susceptible to this type of injury. The application of this preparation to stone fruits is usually considered unsafe, and it cannot be satisfactorily used on the small fruits and many other plants.

A number of theories have been proposed respecting the toxic constituent of the different forms of sulphur, and until recently the problem was a subject of considerable speculation. A complete review of the earlier investigations has been given in a recent paper by Young ('22). This investigator demonstrated that regardless of the form of sulphur employed as a fungicide, whether as a compound or as uncombined sulphur, the lasting toxicity is due to an oxidation product of the sulphur itself. He further proved that the toxic substance is liberated most

[1] A fellowship established by the Crop Protection Institute for the investigation of sulphur as a fungicide.

rapidly from sulphur in a very finely divided state, that is, from colloidal sulphur.

EXPERIMENTAL

Since the experimental evidence gained from laboratory tests, up to the present time, has shown that colloidal sulphur has greater fungicidal value than other forms of sulphur, it was thought important to study the methods of its preparation. The primary object was to perfect a method or methods which would serve as a basis for commercial production of colloidal sulphur. After studying a variety of preparations, those materials which indicated sufficient promise were thoroughly tested for injury on the foliage of a variety of plants growing in the greenhouse. The plants used in these tests were the following: peach, potato, tomato, squash, cucumber, cantaloupe, geranium, tobacco, sweet-potato, and rose. Several of these plants were found to be very susceptible to injury by certain sulphur compounds. In those cases in which injury resulted a further study was made in an effort to eliminate the injurious property. The toxicity of the materials was then tested, using the percentage of germination of spores of a variety of economic fungi as indicators. The Van Tieghem cell or the modified hanging-drop culture method used by Young ('22) was employed in this work. The following organisms were used: *Botrytis Alii*, *B. cinerea*, *Colletotrichum Gossypii*, *Glomerella cingulata*, *Gloeosporium venetum*, *Macrosporium sarcinaeforme*, *Sclerotinia cinerea*, and *Ustilago Hordei*. The spores were taken from cultures 10–15 days old, grown on dextrose-potato agar prepared by the method of Duggar, Severy, and Schmitz ('17), except in the case of *Ustilago Hordei*. The spores of the latter organism were obtained from smutted barley heads, freshly collected in the field.

The culture solution used in the hanging drops and in which the sulphur materials were suspended, except in cases otherwise indicated, was a slightly buffered mixture prepared according to the method of Karrer and Webb ('20) and of Young ('22), as follows: Stock solutions of M/5 mannite in M/10 phosphoric acid and of M/5 mannite in M/5 sodium hydroxide were prepared. Equal quantities of the M/5 mannite-M/10 phosphoric acid were

placed in each of ten flasks and successively increasing proportions of M/5 mannite-M/5 sodium hydroxide were added. The flasks were plugged with cotton, sterilized at 15 pounds pressure for 15 minutes, and allowed to stand for a few hours. Titrations made by the colorimetric method (Clark,'20) showed the mixture to have the following range of hydrogen-ion concentrations: P_H 2.4, 3.4, 4.2, 5.0, 5.4, 5.8, 6.2, 6.8, 7.4, 8.4.

THE PREPARATION OF COLLOIDAL SULPHUR FROM SODIUM THIOSULPHATE AND SULPHURIC ACID

It has been shown by Young ('22)[1] and others that when a saturated solution of sodium thiosulphate (hypo) is mixed with concentrated H_2SO_4, a colloidal sulphur is formed having extremely small particles. In order to free the sulphur from the acid solution, it was coagulated with concentrated NaCl and centrifuged out of suspension. The coagulum was then peptized with water. By repeating the coagulation, centrifuging, and peptizing, a pure sample of colloidal sulphur is obtained—except for traces of the salt. This form of colloidal sulphur was found to have a high fungicidal value, but the method of preparation proved to be impractical for its production on a commercial basis.

There remained one other possible method of remedying the acid-hypo mixture, so that it might be used as a spray; namely, to neutralize it with an alkali. Young ('25) also encountered difficulties in this process. He first used NaOH to neutralize his hypo-acid mixture and found this alkali to be unsatisfactory because the concentration of the electrolyte soon precipitated the soluble sulphur. He later used lime sulphur to neutralize the mixture and prepared the material as follows: Fifteen gallons of saturated hypo were slowly stirred into 5 gallons of concentrated H_2SO_4 in a wooden barrel. The mixture was filtered through a fine screen and the filtrate neutralized with lime sulphur diluted one to three with water. During the addition of the lime sulphur, which was slowly effected, a further quantity of 3 gallons of concentrated hypo was introduced. Analysis of the final mixture

[1] Young gives a review of the earlier investigations upon this method of preparing colloidal sulphur.

showed that 1 gallon contained 10 ounces of soluble sulphur. He suggested that if the mixture could have been centrifuged at this point it would have made an excellent spray. As a result of the conditions, however, the soluble sulphur soon precipitated.

When the hypo and H_2SO_4 are mixed according to the method just described, there is a large quantity of insoluble sulphur formed which is thrown out in the form of a gummy mass. This insoluble mass must be filtered off, as has already been indicated, before further procedures. It appeared logical to suspect that this waste could be prevented to a large extent by the introduction of certain glutinous colloids which might be assumed to act as protective colloids. Likewise, the introduction of such colloids might be assumed to exhibit further protective action by preventing the precipitation of the soluble sulphur when electrolytes are added to neutralize the mixture.

E. von Meyer and Lattermoser (Taylor, '21), in 1897, were the first investigators to recognize that the addition of a very stable sol to a less stable sol, that is, an emulsoid to a suspensoid, prevented the precipitation of the latter by salts. The latter author reached the conclusion that the addition of very stable colloids, such as albumin, gelatin, agar, or gum arabic, to a silver sol, prevented precipitation by electrolytes until the stable colloid is gelatinized. In 1902, Zsigmondy ('09) investigated this "protective" action of colloids quantitatively by means of his gold sol, which in pure aqueous solution is itself very stable, but is also very sensitive to salts. The degrees of protection of various substances, such as gelatin, casein, egg-albumin, dextrin, starches, cane sugar, etc., were expressed by the "gold number." Further discussion in this paper will be limited to the use of protective colloids in the preparation and utilization of spray materials.

Various organic and inorganic substances have been recommended by different investigators for increasing the spreading and adhesive qualities of fungicides and insecticides. Though many of the early investigations dealt primarily with the spreading and adhesive qualities of sprays, it is reasonable to assume that the physical qualities were also improved in many cases.

Glue was one of the first substances used as a spreader. Mil-

lardet and Davis (Moore, '21) in 1885 used glue with Bordeaux mixture in the treatment of mildew of the vine, thinking that glue increased the efficiency of the mixture. Lowe ('96) found that glue added to lead arsenate, in the proportion of 2 quarts to 45 gallons of spray, gave satisfactory results in spreading the spray evenly over the foliage of cottonwood. Surface ('05) used glue to increase the adherence of lime-sulphur spray.

More recently Jones ('19) has obtained a patent covering the use of glue as a stabilizer for oil emulsions so that they will mix with lime-sulphur solutions. Yothers and Winston ('24) state that such substances as casein, skimmed milk powder, gelatin, corn meal, wheat flour, and starches are as effective as glue in rendering oil emulsions miscible with lime-sulphur solutions.

Various kinds of soaps have been very commonly employed as spreaders. Gillette ('90) was one of the first investigators to report the use of soap as a spreader for insecticides, namely, Paris green and London purple. Washburn ('91), Galloway ('92), and Swingle ('94) are other early investigators to recommend the use of soaps.

A little later those interested in the field began to investigate the use of spreaders quantitatively. Van Slyke and Urner ('04) showed by chemical analysis that different kinds of soaps varied greatly in water content. Therefore, it was concluded that the addition of a definite quantity of soap to sprays may or may not increase their spreading qualities.

Vermorel and Dantony ('10) studied the surface tension of soap solutions in relation to their ability to spread over a surface. They state that whether or not the addition of soap to fungicides will increase their spreading qualities depends upon the method used in the preparation of the mixture. Parker ('11) recommended the addition of soap to certain arsenical sprays to retard settling and likewise increase spreading qualities.

Lovett ('18, '20) made a rather extensive study of the use of spreaders with arsenical sprays. He concluded that the ability of a solution to hold arsenate in suspension, while not necessarily a criterion as to its value as a spreader, does indicate a physical quality in the solution much to be desired in a spreader. Various organic substances were tested, such as caseinate, glue, gelatin,

sage tea, soap bark, and starches. He arranged the materials tested in the order of their merit, based on compatibility, efficiency, availability, cost, and ease of preparation, as follows: caseinate, glue, gelatin, and soap bark. A number of inorganic compounds were also tested. Oil emulsion gave very promising results, while other inorganic materials were unsatisfactory.

Moore ('21)[1], in a quantitative study of the spreading and adherence of arsenical sprays, found that the addition of materials similar in chemical constitution to the leaf surface causes the spray mixture to form a continuous film over the foliage. Various protein substances and plant infusions gave effective spreading on leaves with surfaces of cellulose, even when they are strongly cutinized. The suspensions containing small-sized particles were found to adhere better than those of larger-sized particles.

Recently, Brinley ('23) has recommended a method of preparing colloidal lead arsenate. The method consists in precipitating the lead arsenate in the presence of a protective colloid, such as gelatin, by the chemical reaction between lead nitrate and disodium arsenate. He found that the method of mixing and diluting the chemicals was a very important factor in obtaining a solution of greatest stability.

Since most of the evidence in the foregoing references points to organic substances as being more effective than inorganic substances in preserving the stability of solutions, it was thought desirable to test the hypo-H_2SO_4 mixture as prepared with certain glutinous colloids. Thus dextrin, peptone, starch, gelatin, and glue were tested in varying percentages. Dextrin and peptone did not prove of any value in preserving the stability of the solution. While starch possesses certain desirable characteristics, gelatin and glue proved to be more satisfactory. The results obtained with gelatin were slightly less desirable than those obtained with glue. Therefore, considering the materials upon their merits, as to efficiency, cost, and availability, glue should be recommended in preference to gelatin.

The hypo and H_2SO_4 were diluted in a number of ways before mixing and the stability of the resulting solutions was tested as prepared with glue. Likewise the stability of the solution was

[1] A review of the history of spreading is given.

tested as prepared with different proportions of the chemicals. Smaller quantities of the hypo in proportion to the H_2SO_4 gave best results. The most satisfactory results were obtained by introducing the glue immediately after the chemicals were mixed. If the mixture is allowed to stand any length of time before the glue is added, the stability of the solution is lost; and, further, the introduction of glue to either chemical before mixing results in the loss of a considerable quantity of sulphur, the latter being thrown out of solution in an insoluble mass.

Since temperature has been shown to be an important factor in the preparation of other colloidal solutions, as well as sulphur, the effect of employing warm solutions was tested, with thoroughly satisfactory results. Wackenroder ('46) observed that low temperatures caused a large part of the sulphur particles to settle out of solution.

In order to neutralize the hypo-H_2SO_4 mixture, while at the same time preserving the stability of the solution and likewise the toxic property of the colloidal sulphur, it was necessary to employ various alkalies. After preliminary tests, strong alkalies were eliminated as unsatisfactory. Sodium carbonate and some other weak alkalies proved satisfactory. Raffo ('08) used Na_2CO_3 to neutralize a sulphur suspension, made from hypo and H_2SO_4, after the sulphur had been centrifuged out of suspension and again peptized with water. The greater part of the sulphur was precipitated, leaving a stable solution of sulphur containing a little Na_2SO_4. The use of glue prevented, to a large extent, the precipitation when weak alkalies were added.

After testing various methods as indicated in the foregoing discussion, the method which proved most satisfactory was as follows: Fifty gms. of hypo were dissolved in 40 cc. of water and warmed to 40–50° C.; 40 cc. of H_2SO_4, specific gravity 1.84, were measured into a 500-cc. glass cylinder. The warm saturated solution of hypo was added slowly to the H_2SO_4 with occasional stirring. Eighty cc. of warm water (30–40° C.) were immediately added to the mixture, followed by the same quantity of 1 per cent glue solution of the same temperature. The temperature, as indicated in each case, is a very important factor and one on which, to a large extent, the stability of the solution depends.

The mixture was allowed to stand 48 hours, after which time the reaction was adjusted to P_H 4.2 with a saturated solution of Na_2CO_3 and then aerated for 30 minutes. The aeration is necessary to free the solution of traces of SO_2 which is very injurious to the foliage of plants. The addition of water as indicated is a very important step in the process. It changes the physical state of the solution to such an extent that the solution will remain stable for several days without the addition of a protective colloid. In this connection, Sobrero and Selmi (Taylor, '21), in 1850, investigating colloidal sulphur formed by the reactions of SO_2 and H_2S in water, make the following remark: "If water is added to it [colloidal sulphur] it divides up, forming an emulsion from which it does not separate out, even on prolonged standing [several months]."

A saturated solution of dibasic sodium phosphate may be used for neutralizing the mixture. It may be added to the hypo-acid mixture immediately, thereby eliminating the necessity of allowing the mixture to stand 48 hours before adjusting the reaction.

After the two solutions were thoroughly tested on the foliage of plants, toxicity tests were made. The method employed in preparing the sulphur suspensions for testing was essentially the same as that used by Young ('22) in his toxicity studies, and was as follows: Three test-tubes were provided with pipettes that extended through the cork stoppers to the bottom of the tubes. By this means drops could be transferred readily to the hanging-drop cells. Ten cubic-centimeters of the slightly buffered solution, P_H 4.2, were added to each tube. This gave a duplicate series, one tube each for the two sulphur suspensions to be tested and one for the control culture. One cubic centimeter of the sulphur suspensions was added to the tubes.

The technique of planting the hanging-drop cultures was the same as that employed by Webb ('21) in his germination studies and later slightly modified by Young ('22), and was as follows: Ground-glass rings were cemented to glass slides by means of parawax and petrolatum. Two of these rings were placed on each slide and 3 slides constituted a series for each organism. A large drop of the sulphur suspension to be tested for toxicity

was placed in the bottom of the two cells. Another drop was placed on each slide and three slides constituted a series for each organism. A large drop of the sulphur suspension to be tested for toxicity was placed in the bottom of the two cells. Another drop was placed on a clean sterile glass slide. A definite spore suspension was made in the drop. By means of a small sterile glass rod a small part of the drop was transferred to a clean sterile cover-glass and spread out in the form of a smear. The cover-glass was inverted and sealed air-tight by means of petrolatum over the glass cell. The cultures were incubated at 23° C. and germination counts made at the end of 18 hours. The results are recorded in table I.

TABLE I

TOXICITY OF CERTAIN FORMS OF COLLOIDAL SULPHUR. THE FIGURES INDICATE PERCENTAGE OF GERMINATION[1]

Form of sulphur	Organism				
	Botrytis cinerea	Colletotrichum Gossypii	Glomerella cingulata	Macrosporium sarcinaeforme	Sclerotinia cinerea
Control—without sulphur	80	65	76	94	60
Colloidal sulphur neutralized with Na_2CO_3	5	0	8	44	2
Colloidal sulphur neutralized with Na_2HPO_4	15	4	12	57	5

[1] Average of duplicate tests run at two different times.

The results in this table show that both colloidal sulphur solutions tested were toxic to all the organisms used. The solution prepared with disodium phosphate was slightly less toxic than that prepared with Na_2CO_3. This can be accounted for in that it required more of the phosphate to neutralize the mixture. Therefore, there was less sulphur in the mixture.

THE PREPARATION OF COLLOIDAL SULPHUR FROM LIME SULPHUR

Since the introduction of lime sulphur as an orchard spray serious injuries to foliage of plants have been frequently reported as a result of its use. Injury is often more frequent when the spray is used in combination with certain arsenicals. This is shown by a survey of the literature from various agricultural experiment stations. A number of methods for the elimination of these difficulties have been suggested by different investigators, all of which relate primarily to the method of diluting, mixing, and applying the spray, or to the time of application, or the evidence of inferior compounds. Since lime sulphur is composed largely of sulphides (pentasulphides) the caustic action on foliage of plants has been attributed to these soluble compounds. A few investigators have suggested that the injurious properties can be eliminated by precipitating the soluble sulphides with salts or acids. These methods, however, have not come into general use.

Wallace ('10), in a study of lime-sulphur spray injury, precipitated commercial lime sulphur, diluted 1–30 with water, by applying it with a carbonic-acid gas sprayer. The solution was allowed to stand in contact with the gas one-half hour before the application of the spray. A heavy white precipitate was formed, and this did not cause injury to apple and peach foliage when used alone as a spray. However, when lead arsenate was introduced into the mixture before the precipitation, the resulting material used on young peach trees caused almost complete defoliation.

Stewart ('12), in a similar study, pointed out that lime-sulphur-arsenical injury could be eliminated by precipitating the mixture with iron sulphate, or "copperas," added at the rate of 3½ pounds to 50 gallons of 1.01 spray solution. The mixture was found to be very disagreeable to handle, due to its tenacious and blackening qualities. Also serious discoloration of fruit resulted from its use, and the effectiveness of arsenicals was reduced. Waite ('10) had previously reported the use of iron sulphate and copper sulphate in the preparation of self-boiled lime sulphur.

Safro ('13), after a study of lime sulphur spray injury, con-

cluded that the effect was due to the soluble sulphide content of the spray. He found that the injury could be reduced by rendering the sulphides insoluble, that is, by precipitating them. His results indicated that the injury could be reduced by the addition of either iron, copper, or zinc sulphate, the salt being first dissolved in water, in the proportion of 4 pounds to 100 gallons of diluted (1 to 20) lime-sulphur solution. Carbon dioxide and H_2SO_4 were also used, the substances being introduced until the remaining sulphides gave the spray an amber color. The carbon dioxide entirely eliminated the injury.

More recently, definite methods have been given for precipitating lime sulphur for commercial use. Ramsay and Cooke ('22) describe a method of preparing colloidal sulphur from lime sulphur which has been used effectively as a spray in Australia. The method recommended is as follows: Ten gallons of home-made lime sulphur, 26° Baumé, were diluted with 25 gallons of water in a barrel of 40 or 50 gallons capacity. Using a porcelain or earthenware vessel, 6 pints of strong commercial H_2SO_4 were poured slowly into 9 pints of cold water and the solution allowed to cool. The cold diluted H_2SO_4 was then added to the diluted lime sulphur, 2 or 3 ounces at a time, stirring well after each addition of acid, until the typical yellow color of the original lime-sulphur disappeared and until the addition of H_2SO_4 produced no further precipitation of sulphur. The precipitated sulphur was allowed to settle for a day or two and the clear liquid above was siphoned or decanted off. Three pounds of cheap glue were dissolved in just sufficient hot water to render the glue soluble, and, while still hot, stirred thoroughly into the precipitated sulphur. For use, the mixture thus obtained was diluted with water to 250 gallons. This gave approximately 5 pounds precipitated sulphur per 100 gallons of spray.

After making a study of the toxicity of the various ingredients of decomposed lime sulphur, Young ('22) showed conclusively that the lasting toxicity was due to the precipitated sulphur. This form of precipitated sulphur was found to be about as toxic to parasitic fungi as "hydrophobic" colloidal sulphur. Later ('25) he prepared colloidal sulphur from lime sulphur as a summer spray. The results from the use of this material in

combating apple scab were very encouraging. His method was as follows: Ten gallons of lime sulphur, 32° Baumé, were placed in a 50-gallon barrel. To this were added 10 gallons of water and 1 pound of glue, the latter previously dissolved in hot water. Sulphuric acid diluted with 3 parts of water was then added until the reaction of the mixture was P_H 4.2. The mixture was allowed to settle and the supernatant liquid decanted off. The mixture contained about 1 pound of active sulphur per gallon. Five gallons of the precipitate were diluted with water to 95 gallons before using as a spray.

Since the recent uses of precipitated lime sulphur as a spray have given encouraging results, it was considered important to make further studies upon its preparation with the object of producing a mixture of greater stability. Likewise from previous tests of toxicity (Young, '22), the mixture would be expected to exhibit greater fungicidal properties. There were several factors which were thought to be important in the preparation, namely: (1) the choice of acids and the dilution of these; (2) the concentration and method of diluting the lime-sulphur solution; (3) the temperature; and (4) the protective colloid, its concentration, and the method of introduction.

The lime sulphur used for this work was a freshly made solution, prepared according to the Van Slyke method, that is, in the proportion of 80 pounds sulphur, 36 pounds lime (CaO), and 50 gallons of water, these ingredients being boiled together one hour. Preliminary tests were made with a number of acids and phosphates to determine which of those were most desirable for precipitating the freshly made lime sulphur. The use of phosphates, of certain acids, and also of strong solutions of acids gave unsatisfactory results and these procedures were eliminated. Weak solutions of HNO_3 and H_2SO_4 gave very promising results. Glue, which proved to be the most satisfactory in the previous preparations, was used as a protective colloid. The concentration of the glue, the method of introducing it, the dilution of the lime sulphur, and the temperature of the solutions employed in the preparation were found to be the important factors upon which, to a large extent, the stability of the solution depended.

Since the preliminary tests showed that the dilution of the

acid used in precipitating lime sulphur had an important bearing upon the stability of the solution, further tests were made in order to determine what strength of HNO_3 and H_2SO_4 could be most satisfactorily employed. Twenty-five cc. of lime-sulphur solution were measured into each of 10 glass beakers of 300 cc. capacity. The beakers were numbered 1–5 inclusive, which gave a duplicate set, one set each for the two acids to be tested. The mixtures were then adjusted to the reaction P_H 4.2, with dilutions of HNO_3 and H_2SO_4 as follows:

No. 1. Acids diluted 1–3 with water.
No. 2. Acids diluted 1–5 with water.
No. 3. Acids diluted 1–10 with water.
No. 4. Acids diluted 1–20 with water.
No. 5. Acids diluted 1–40 with water.

The resulting solutions showed a striking range in stability. The precipitated sulphur in solutions No. 1 settled out of suspension within a few hours after it was prepared. Solutions No. 2 remained stable several days. Solutions No. 3 remained stable about 2 weeks. Solutions No. 4 remained stable several months. Solutions No. 5 were less stable than No. 3. The solutions prepared with HNO_3 possessed slightly greater stability in all cases than those prepared with H_2SO_4. These results show conclusively that the strength of the acid used in precipitating the sulphur is an important factor in producing a product of the desired stability.

After determining the proper dilution of acid to use, further studies were made of different procedures in making the preparation. The method which proved the most satisfactory is as follows: Fifty cc. of freshly made lime sulphur were measured into a glass beaker of 600 cc. capacity. One gram of dry flake glue was dissolved in 200 cc. of water and mixed with the lime sulphur while warm (35–40° C.). Ten cc. of HNO_3 were diluted with water in a separate container. The diluted HNO_3 was then added to the diluted lime-sulphur mixture very slowly, with stirring, until the reaction P_H 4.2 was obtained. The mixture was allowed to set for several hours in the open container, with occasional stirring, to remove the H_2S. This gave a final dilution, in respect to the original lime-sulphur solution, of 1 to 9.

The mixture contained approximately 1.8 per cent sulphur. Colloidal sulphur prepared by this method will remain stable for several months. Sulphuric acid diluted 1–20 may be used to precipitate the lime sulphur mixture. However, as previously indicated, the solution as prepared with H_2SO_4 is less stable.

In determining the toxicity of this form of colloidal sulphur, the mixtures prepared with HNO_3 and H_2SO_4 were compared with lime sulphur precipitated by the CO_2 in the air, the latter being effected as follows: Ten cc. of lime sulphur solution were diluted with 60 cc. of water and this solution was poured into a large open dish and allowed to decompose. The precipitate was then taken up in 70 cc. of water. One cc. of each of these 3 precipitated lime-sulphur solutions was then added to 5 cc. of the slightly buffered mannite-H_2PO_4-NaOH mixture of P_H 4.2 (except in tests with *Macrosporium sarcinaeforme*, where M/5 mannite was used), and the toxicity determined as before. The organisms used and the results are recorded in table II.

TABLE II

TOXICITY OF PRECIPITATED LIME-SULPHUR SOLUTIONS. THE FIGURES INDICATE THE PERCENTAGE OF GERMINATION (AVERAGE OF CULTURES RUN AT 2 DIFFERENT TIMES)

Form of sulphur	Botrytis cinerea	Organism			
		Glomerella cingulata	Macrosporium sarcinaeforme	Sclerotinia cinerea	Ustilago Hordei
Control—without sulphur	84	64	88	59	73
Lime sulphur solution precipitated with HNO_3	24	10	10	0	1
Lime sulphur solution precipitated with H_2SO_4	55	13	30	0	3
Lime sulphur solution precipitated by CO_2 in air	71	—	—	—	21

From the results exhibited in table II it is clear that the toxicity of the sulphur prepared by the different methods is related to

the stability of the sulphur suspensions. The sulphur precipitated from lime sulphur by the CO_2 in the air settles out of suspension in a very short time, and is therefore the least toxic of the three forms of sulphur tested. The solution prepared with HNO_3 possesses the greatest stability and likewise exhibits the greatest toxicity.

The mixtures prepared with HNO_3 and H_2SO_4 were thoroughly tested on the foliage of plants already named growing in the greenhouse. They were found to spread over the surface of the foliage evenly and adhere well; in fact it was difficult to remove the films by washing with a strong stream of water from the hose.

PREPARATION OF COLLOIDAL SULPHUR BY MEANS OF SO_2 AND H_2S

The formation of colloidal sulphur by the reaction between SO_2 and H_2S in water was first recorded by Berthollet (1798). The reaction was later studied by Wackenroder ('46) who discovered pentathionic acid and prepared it by passing H_2S into a saturated solution of SO_2, always keeping an excess of the latter. Lewes ('81), Spring ('82), Shaw ('83), and Débus ('88) are other early contributors to the study of the properties of pentathionic acid formed by this process. The reaction of SO_2 and H_2S has been studied more recently by Riesenfeld and Feld ('21) in a study of polythionic acid and polythionate.

Selmi and Sobrero ('50) (also earlier investigators) made rather extensive studies of colloidal sulphur formed by the reaction of SO_2 and H_2S. A review of the work is given by Odén ('13). That there is more than one state of colloidal sulphur formed by this process is shown by Selmi ('52) who prepared colloidal sulphur as follows: Sulphur dioxide was passed into distilled water until a saturated solution was formed. Hydrogen sulphide was then passed into the saturated solution of SO_2, care being taken not to have an excess of H_2S, as it precipitates the soluble sulphur. The solution was then centrifuged to remove the larger particles and the supernatant liquid coagulated with sodium chloride. The coagulum was then peptized in water. Further evidence of the two forms of colloidal sulphur is given in the papers cited above. Young ('22) has designated the two forms, according to their degree of hydration, as hydrophilic

and hydrophobic colloidal sulphur. There are in all probability several grades of fineness in the hydrophobic forms. This is shown in the work of Odén ('13).

The colloidal sulphur prepared from SO_2 and H_2S in this investigation consists largely of the hydrophobic form. The method used was as follows: A rubber stopper having two holes was provided with two thistle tubes, over the mouths of which finely perforated parchment paper was securely fastened. The tubes were inverted into a 2-liter flask containing 1500 cc. of water, the stopper closing the mouth of the flask. Sulphur dioxide (compressed liquid) and H_2S (made from FeS and 1 to 1 muriatic acid) were passed simultaneously into the water through the thistle tubes, keeping the sulphur dioxide slightly in excess. After three hours a slight excess of H_2S was introduced to free the solution of SO_2. The suspension thus formed contained 12 per cent sulphur, which had great stability. In fact, there was but very little settling out after standing three months.

The efficiency of the method is determined by the fineness of the perforations through which the gases are introduced into the liquid. In the method described the gases are introduced in the form of a fine spray, which permits a more thorough mixing in the solution. The introduction of the gases in the form of large bubbles was found to be inefficient and unsatisfactory in preliminary tests. The use of distilled water has been found more desirable than tap water, especially since the latter may contain sufficient salts or alkalies to effect a partial precipitation of the colloidal sulphur as it is formed.

The colloidal sulphur solution thus prepared has an acid reaction slightly beyond the acid range of available indicators, P_H 1.0. Wackenroder ('46) found that some H_2SO_4 was formed during the process in the presence of oxygen. It was later found difficult to free the mixture entirely of SO_2, and it was evident that traces were left in the solution. The solution was found to be injurious to the foliage of plants; therefore, it was necessary to adjust the reaction, or treat the solution in some way before it could be used as a spray.

The weak alkalies used for adjusting the colloidal sulphur

solution prepared by the hypo-H_2SO_4 method, namely, Na_2CO_3 and the Na_2HPO_4, caused precipitation of the colloidal sulphur when freshly prepared by this method. Similar results were also produced by the introduction of glue or gelatin. However, promising results were obtained by aerating the mixture for 48 hours and then adjusting the reaction to P_H 4.2 with a weak solution of Na_2HPO_4. The resulting solution remained stable for 3 months, after which time the colloidal sulphur was precipitated. This material was found to be very toxic to fungi when first prepared. It proved effective for control of carnation rust in the greenhouse.

Later the colloidal sulphur was coagulated with NaCl and allowed to settle out of solution. The method was as follows: A saturated solution of NaCl was added to the freshly prepared colloidal sulphur suspension until it changed from a bright to a pale yellow color. The mixture was allowed to set two hours, after which time the coagulum had settled out of suspension. The supernatant liquid was then siphoned off and the coagulum washed with water and allowed to settle again. The supernatant liquid was again siphoned off. The sulphur is left in the form of a thin paste, containing about 70 per cent water and a little NaCl. The sulphur readily assumes the colloidal state when further quantities of water are added. Selmi and Sobrero ('50) found that colloidal sulphur prepared from SO_2 and H_2S could be coagulated by a neutral sodium salt, and again be emulsified in water. They state that when potassium salts are used the precipitated sulphur completely loses the property of emulsifying in water.

When the sulphur paste (prepared by the above method) is resuspended in water, shortly after it has been coagulated, the suspension remains stable over a long period of time. In fact, such a suspension was kept in the laboratory for 8 months, and after this there was still little or no settling of the sulphur. However, if the coagulum is not taken up in water, the sulphur begins to crystallize in about 1 month and soon loses its colloidal property. The paste form would be much more desirable for practical use. Therefore further investigations should be made to determine if it can be treated in such way as to prevent crystallization.

The results on the toxicity tests of this form of colloidal sulphur are discussed on subsequent pages.

THE TOXICITY OF CERTAIN FORMS OF COLLOIDAL SULPHUR IN RELATION TO THE HYDROGEN-ION CONCENTRATION

Since it was shown by Young ('22) that the hydrogen-ion concentration has a marked influence upon the toxicity of certain forms of colloidal sulphur, it was thought important to determine if this influence may be exhibited by other forms of these colloidal preparations. A colloidal sulphur solution was prepared from SO_2 and H_2S as already described, with modifications as follows: After the colloidal sulphur had been coagulated with NaCl, the solution was then centrifuged for 15 minutes at 1500 revolutions per minute. The supernatant liquid was then decanted. Upon drying and weighing, the coagulum was found to contain 50 per cent of sulphur.

One gm. of the coagulum was weighed out and taken up in 100 cc. of distilled water. This gave a dilution of sulphur of 0.5 gm. in 100 cc., or 1 to 200. When freshly made, the reaction of the solution was P_H 4.0; after standing in a closed container for 1 week the P_H had changed to 2.2. The freshly made sulphur suspension also changed the hydrogen-ion concentrations of the culture solutions. Therefore it was found necessary to adjust the sulphur suspensions to the range of hydrogen-ion concentrations corresponding to the particular hydrogen-ion concentration of the culture solution, which was done as follows: Fifty-cc. quantities of the 1 to 200 colloidal sulphur suspension of P_H 4.0 were measured into each of 11 clean glass beakers. The suspensions were then adjusted toward and into the alkaline range with an increasing number of drops of M/10 Na_2HPO_4 solution, and into the acid range with M/10 H_3PO_4 solution. This gave a range of hydrogen-ion concentrations as follows: P_H 2.4, 3.4, 4.2, 5.0, 5.4, 5.8, 6.2, 6.8, 7.4, and 8.4. The colloidal sulphur suspension thus prepared did not change the hydrogen-ion concentrations of the culture solution during the 18-hour period of incubation of the cultures. The stability of the sulphur suspensions of different hydrogen-ion concentrations will be discussed later.

In later work it was found that if the coagulation, centrifuging, and peptizing with water is repeated 3 times, the hydrogen-ion concentration of the resulting colloidal sulphur suspension does not change even on prolonged standing. Likewise, such a suspension does not evert the hydrogen-ion concentration of the culture solution. This is probably due to the buffer action of the phosphate which was added.

The culture solution used for making the toxicity studies was the slightly buffered mannite-H_3PO_4-NaOH mixture prepared according to the method already described. Twenty test-tubes were provided with pipettes that extended through the cork

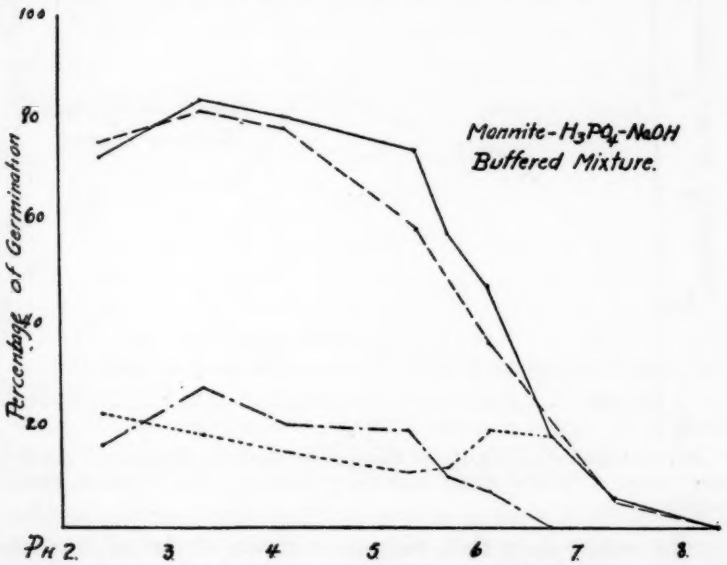

Fig. 1. Germination of spores of *Botrytis cinerea* in hanging-drop cultures: toxic action of certain forms of colloidal sulphur; prepared by method 1— · — · —; prepared by method 1, crystallized — — —; prepared by method 2 - - - - -; control, without sulphur————.

stopper to the bottom of the tubes, according to the method employed by Young ('22). This constituted a duplicate series of 10 each, and 10 cc. each of the slightly buffered solutions were added to the tubes so that each tube represented a particular

hydrogen-ion concentration. The colloidal sulphur suspensions were added to one series, with accurately calibrated pipettes, in quantities to make the dilutions indicated in table III.

The hanging-drop cultures were prepared in closed-ring cells according to the method already described, and were incubated at 23° C. After germination of 18 hours, counts were made. The results of these tests are shown in table III, also in figs. 1–4.

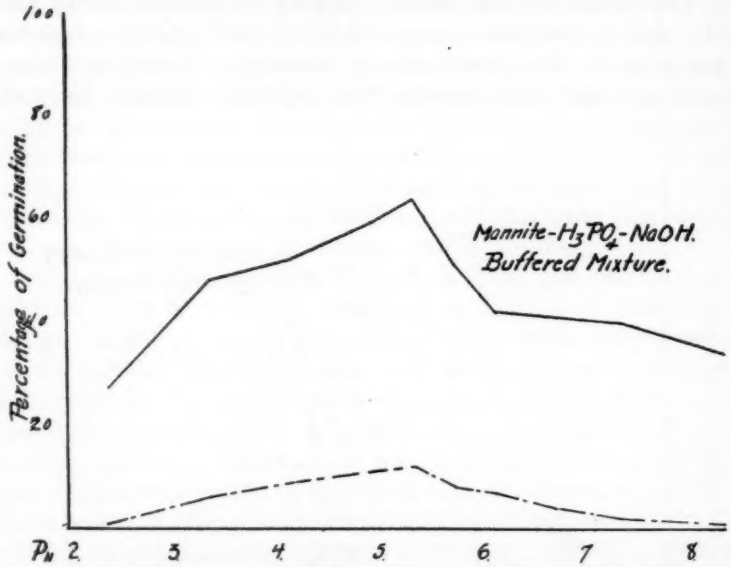

Fig. 2. Germination of spores of *Gloeosporium venetum* in hanging-drop cultures: toxic action of colloidal sulphur prepared by method 1 — - —; control, without sulphur ———.

The results show that the concentration of the sulphur employed in the culture solutions was a very important factor. The organisms tested vary greatly in their resistance to the toxicity of this form of colloidal sulphur, *Macrosporium sarcinaeforme* being very resistant, while extremely weak dilutions are sufficient to kill *Sclerotinia cinerea*. In general, this form of sulphur is most toxic toward, and in, the alkaline range of hydrogen-ion concentrations. The highest percentage of germination was in most cases at P_H 3.4. Beyond this point toward the

alkaline range there was a gradual decrease to the point P_H 5.8, after which there is a sharp decrease.

The influence of the hydrogen-ion concentration upon the toxicity of this form of colloidal sulphur differs from the results obtained by Young ('22) with his hydrophilic colloidal sulphur. In order to make a comparison by the same method, hydrophilic

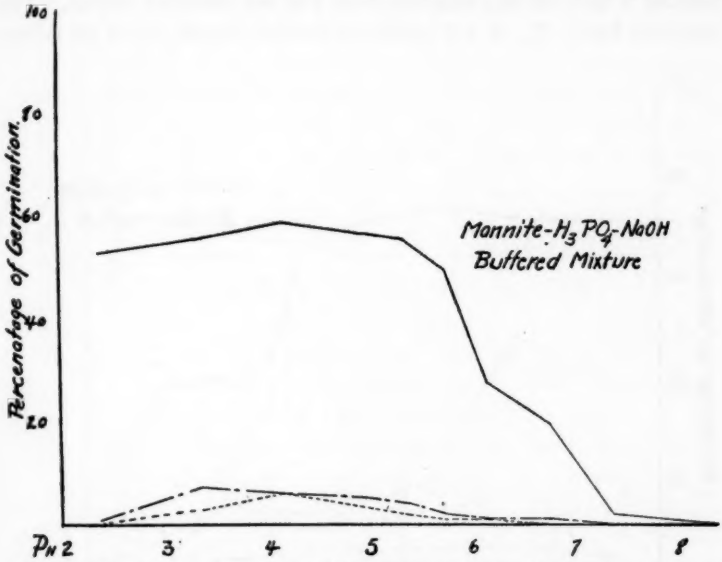

Fig. 3. Germination of spores of *Sclerotinia cinerea* in hanging-drop cultures: toxic action of certain forms of colloidal sulphur; prepared by method 1 — · —; prepared by method 2 - - - - - -; control, without sulphur ————.

colloidal sulphur was prepared according to the method of Young ('22) which was as follows: Fifty gms. of pure crystalline sodium thiosulphate were dissolved in 30 cc. of distilled water; 70 gms. of concentrated H_2SO_4, sp. gr. 1.84, arsenic-free, were weighed into a glass cylinder of 300 cc. capacity. The cylinder was placed in a vessel of cold water, and the saturated solution of sodium thiosulphate added very slowly with occasional stirring. The mixture was then allowed to cool, and 30 cc. of distilled water were added. The preparation was then placed in a water bath and warmed to 80° C. for 10 minutes and filtered through glass

wool to remove insoluble sulphur. After the mixture was allowed to cool, concentrated NaCl was added and the mixture centrifuged for 30 minutes at 1500 revolutions per minute. The coagulum was then peptized with 100 cc. of distilled water and the insoluble sulphur centrifuged out. The peptized sulphur was then treated 3 times with 25 cc. of saturated NaCl and finally peptized by adding 1 gm. of the coagulum to 100 cc. distilled water. The solution had a P_H of 4.2 ,which does not change upon standing.

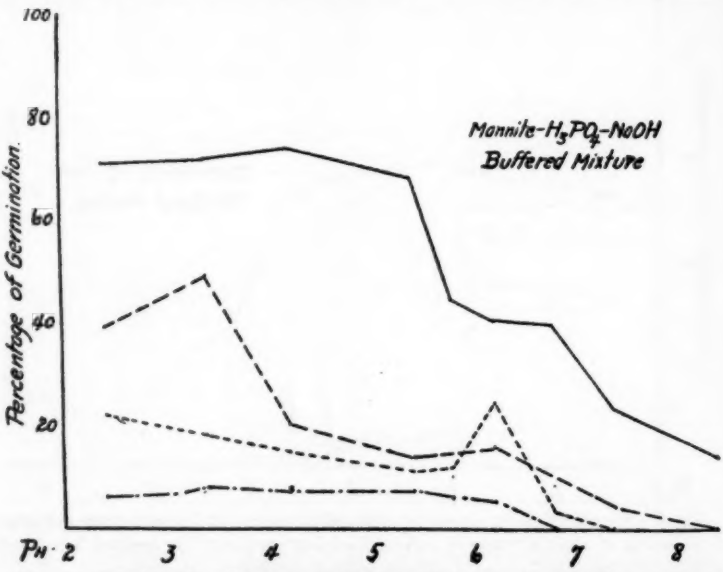

Fig. 4. Germination of spores of *Ustilago Hordei* in hanging-drop cultures: toxic action of certain forms of colloidal sulphur; prepared by method 1 - — - —; prepared by method 1, crystallized — — —; prepared by method 2 - - - -; control, without sulphur ———.

By means of an accurately calibrated pipette, aliquot parts were measured into each of 10 tubes containing the slightly buffered solution, to make the dilutions indicated in table III. The hanging-drop cultures were made in exactly the same way as described for the other forms of colloidal sulphur, and germination counts were made after an incubation interval of 18 hours at 23° C. The results are shown in table III and in figs. 1, 3, and 4.

TABLE III

TOXICITY OF CERTAIN SULPHUR SUSPENSIONS. THE FIGURES REPRESENT PERCENTAGE OF GERMINATION (AVERAGE OF 2–5 TESTS)

Organism	Form of colloidal sulphur	Dilution of sulphur	Hydrogen-ion concentration (P_H)									
			2.4	3.4	4.2	5.0	5.4	5.8	6.2	6.8	7.4	8.4
Botrytis Alii	Control, without sulphur		59	81	82	71	70	51	38	8	2	0
	Prepared by Method 1	1–2,000	9	14	12	9	5	2	1	0	0	0
	Prepared by Method 2	1–2,000	0	0	0	0	0	0	0	0	0	0
Botrytis cinerea	Control, without sulphur		72	83	80	—	74	57	47	18	6	0
	Prepared by Method 1	1–2,000	16	27	20	—	19	10	7	0	0	0
	Prepared by Method 1, sulphur crystallized	1–1,000	75	81	78	—	58	—	36	—	5	0
	Prepared by Method 2	1–4,000	0	0	0	—	0	—	0	—	0	0
		1–8,000	22	18	15	—	11	12	19	18	6	0
Colletotrichum Gossypii	Control, without sulphur		48	59	65	—	78	54	40	22	13	7
	Prepared by Method 1	1–10,000	2	15	16	—	16	17	14	14	9	4
Gloeosporium venetum	Control, without sulphur		27	48	52	59	64	51	42	41	40	3.4
	Prepared by Method 1	1–2,000	0	0	0	2	0	0	0	0	0	0
		1–4,000	1	6	9	11	12	8	7	4	2	1
	Prepared by Method 2	1–4,000	0	0	0	0	0	0	0	0	0	0

TABLE III (Cont'd.)

Organism	Form of colloidal sulphur	Dilution of sulphur	Hydrogen-ion concentration (P_H)									
			2.4	3.4	4.2	5.0	5.4	5.8	6.2	6.8	7.4	8.4
Macrosporium sarcinaeforme	Control, without sulphur		17	90	94	97	94	93	93	90	83	75
	Prepared by Method 1	1–500	1	21	22	25	29	27	27	18	19	20
Sclerotinia cinerea	Control, without sulphur		53	56	59	57	56	50	28	20	2	0
	Prepared by Method 1	1–10,000	0	0	0	0	0	0	0	0	0	0
		1–20,000	1	7	6	5	4	2	1	1	0	0
	Prepared by Method 2	1–20,000	0	3	6	—	2	1	1	0	0	0
Ustilago Hordei	Control, without sulphur		71	72	73	—	68	44	40	39	23	14
	Prepared by Method 1	1–1,000	6	8	7	—	7	6	5	0	0	0
	Prepared by Method 1, sulphur crystallized	1–1,000	39	49	20	—	14	—	16	—	4	—
	Prepared by Method 2	1–2,000	22	18	15	—	11	12	24	3	0	0

In the table and figures just referred to, the form of colloidal sulphur is indicated according to the method of its preparation. Thus, Method I refers to the colloidal sulphur prepared by the SO_2-H_2S process, and Method 2 refers to the hydrophilic form of colloidal sulphur prepared with sodium thiosulphate and H_2SO_4.

The results of the toxicity tests of the two forms of colloidal sulphur show that Young's hydrophilic form is much more toxic to the organisms used, as it required much smaller quantities of this form to inhibit germination of spores. This toxicity corresponds to the size of the sulphur particles. It has already been indicated that the form prepared by the SO_2-H_2S method is largely the hydrophobic form with probably a small amount of the hydrophilic form.

The influence of the hydrogen-ion concentration upon the hydrophilic colloidal sulphur was found to be essentially the same as that recorded by Young ('22). Upon examining the culture tubes containing the hydrophilic colloidal sulphur, it was also found that settling out was rapidly increased as the P_H increased beyond P_H 5.4. However, this does not explain the difference in toxicity of the two forms. It appears logical that if the toxic substance, exhibited by the colloidal sulphur prepared by the SO_2-H_2S method, is pentathionic acid ("truly 'soluble' sulphur") it would be precipitated at a higher P_H than 5.4, as has been shown to be the case with hydrophilic colloidal sulphur. Further proof that pentathionic acid would be precipitated, particularly in the more alkaline solution, is shown by the investigations of Wackenroder ('46), Shaw ('83), Débus ('88), and others. Therefore, it is shown conclusively that the toxic substance exhibited by the form of colloidal sulphur prepared from SO_2 and H_2S possesses properties different from the toxic substance exhibited by the hydrophilic form, prepared according to the method of Young. From the evidence, the latter is, in all probability, pentathionic acid.

There were some indications that the toxic substance exhibited by the colloidal sulphur prepared from SO_2 and H_2S may be destroyed by repeating the coagulation, centrifuging, etc., several times. This remains to be established by more detailed study.

It was later found that when colloidal sulphur was prepared by the SO_2-H_2S method, coagulated with NaCl, and kept for some time in coagulated form, the sulphur soon crystallized and would no longer remain in suspension when water was added. Suspensions were made of this crystallized sulphur and tests were made in the usual way with *Botrytis cinerea* and *Ustilago Hordei*. The results of these tests, in table III, show that the toxic substance has been destroyed to a large extent.

THE INFLUENCE OF THE HYDROGEN-ION CONCENTRATION UPON THE STABILITY OF COLLOIDAL SULPHUR SUSPENSIONS, AND THE RESULTING EFFECT UPON THE TOXICITY

Since it was shown in the toxicity tests that the form of colloidal sulphur prepared by the reaction of SO_2 and H_2S exhibits its greatest toxicity at a higher P_H than 4.2, it was considered important to determine whether the hydrogen-ion concentration has any influence upon the stability of the suspension. A colloidal sulphur dispersion was prepared by the SO_2-H_2S method, coagulated with NaCl and centrifuged. The coagulum was then weighed and taken up with distilled water, in the proportion of 1 gm. of sulphur to 200 cc. of water. Fifty-cc. portions of the suspension were measured into each of 11 beakers. The P_H of the suspension was 4.2. The suspensions were then adjusted towards, and into, the alkaline range with M/10 Na_2HPO_4, and on the acid side with M/10 H_3PO_4. The resulting preparations had the following range of hydrogen-ion concentrations: P_H beyond the acid range, 2.4, 3.2, 4.2, 5.0, 5.4, 5.8, 6.2, 6.8, 7.4, and 8.4. The acid solution was adjusted slightly beyond the acid range of available indicators. Portions of these solutions were transferred to clean test-tubes, and the tubes were closed with corks. The remaining portions were used in the subsequent experiment.

The suspensions showed a very striking range in stability. Settling out of the sulphur was increased rapidly as the P_H increased beyond 5.8. A photograph of the tubes was made after 8 months and is shown in pl. 20. The stability of the preparations was as follows: P_H 8.4 began to aggregate and settle in about 1 week and had settled out completely in 2 weeks. P_H 7.4

settled out in 3 weeks. P_H 6.8 settled out in 4 weeks. P_H 6.2 had settled out to a large extent after 7 weeks. P_H 5.8 changed from a bright yellow to cream-white in about 8 weeks, with some settling out. After 8 months this solution had largely settled, but the photograph does not bring this out clearly because of sulphur particles sticking to the sides of the tubes, which could not be removed without agitation of the solution. The acid solution (that is, the solution which was adjusted beyond the acid range of available indicators) began to settle in about 1 month and continued slowly. There was still some sulphur in the suspension after 8 months, as shown in pl. 20. No great difference was manifest in the stability of the preparations represented by P_H 2.4, 3.2, 4.2, 5.0, and 5.4. There was some sediment in all the tubes after 8 months, but the range from P_H 4.2 to 5.4 shows greatest stability.

The tubes were shaken, and 0.5 cc. were taken from each tube and transferred to tubes containing 10 cc. of the slightly buffered solution of like hydrogen-ion concentration. This gave a final concentration of sulphur of 1 to 4,000 which is weaker than that used for other toxicity tests. Spore suspensions of *Botrytis cinerea* were made from the sulphur suspensions, and germination tests were made in the usual way in closed-ring cells. The results are shown in table IV.

TABLE IV

TOXICITY TESTS WITH CERTAIN FORMS OF COLLOIDAL SULPHUR. THE FIGURES INDICATE PERCENTAGE GERMINATION

Organism	Form of sulphur	Hydrogen-ion concentration (P_H)							
		2.4	3.2	4.2	5.4	5.8	6.2	6.8	7.4
Botrytis cinerea	Control, without sulphur	72	83	80	75	57	47	18	6
	Colloidal sulphur adjusted to P_H range indicated after standing 8 months	24	60	67	47	62	39	29	10

The results of this test show that when the stability of the sulphur is destroyed by a higher P_H than 5.4, the toxic substance is likewise destroyed. In this case there was still some sulphur

in suspension at P_H 5.8. However, it did not exhibit any toxicity to *Botrytis cinerea*. The suspensions referred to as P_H 2.4, 3.2, 4.2, and 5.4 are all toxic even with the very weak concentration of sulphur used.

THE TOXICITY OF VOLATILE PRODUCTS OF COLLOIDAL SULPHUR

Since it was demonstrated by Young ('22) that hydrophilic colloidal sulphur exhibited the usual degree of toxicity when the spores were not in direct contact with the sulphur, it was thought important to determine whether the toxic substance of colloidal sulphur made by the SO_2-H_2S method would be liberated to the same extent. To test this point, portions of the freshly prepared sulphur suspensions, employed in the preceding experiment, were used. These suspensions had been adjusted, as indicated, to the hydrogen-ion concentration range P_H 2.4–8.4.

The organisms used were *Botrytis Alii* and *Ustilago Hordei*. The hanging-drop cultures were made in the usual way, with modifications as follows: A small quantity (one-tenth cc.) of each sulphur suspension was placed in the bottom of each cell. The spores were suspended in the slightly buffered mixture without sulphur. In this way the spores were not in direct contact with the sulphur particles, being separated by the height of the cell, which was 8 mm. The cultures were incubated at 23° C. and germination counts made at the end of 18 hours. The results are given in table v.

TABLE V

TOXICITY OF VOLATILE PRODUCTS FROM CERTAIN COLLOIDAL SULPHUR SUSPENSIONS. THE FIGURES REPRESENT PERCENTAGE GERMINATION

Organism	Form of sulphur	Hydrogen-ion concentration							
		2.4	3.2	4.2	5.4	5.8	6.2	6.8	7.4
Botrytis Alii	Control, without sulphur	59	81	82	70	51	38	8	2
	Colloidal sulphur in bottom of cells	2	5	7	6	4	0	0	0
Ustilago Hordei	Control, without sulphur	71	72	73	68	44	40	39	23
	Colloidal sulphur in bottom of cells	3	19	17	14	12	11	10	4

The results in this table are similar to those recorded in table III. The colloidal sulphur exhibits the usual degree of toxicity, that is, the toxicity was greatest beyond P_H 4.2, with freshly prepared suspensions.

THE EFFECT OF DRYING AND AERATING UPON THE TOXICITY OF COLLOIDAL SULPHUR

Since some growers prefer to use sulphur dusts, rather than spray solutions, a test was made to determine whether colloidal sulphur could be dried without destroying its stability and toxic property. A colloidal sulphur suspension was prepared by the SO_2-H_2S method, was coagulated with NaCl, and centrifuged. One gm. of the coagulum was weighed on to a clean watch-glass, spread over the surface, and allowed to dry thoroughly at room temperature. After 5 days the sulphur was in the form of a very fine powder. The dry sulphur was taken up with 200 cc. of distilled water. The sulphur assumed the colloidal state readily and the resulting suspension had a reaction of P_H 5.0. Toxicity tests were made in the usual way, and the results are recorded in table VI.

Two series of wash bottles were arranged to test the effect of aeration upon the stability and toxicity of colloidal sulphur. Freshly made colloidal sulphur preparations containing 1 gm. of sulphur in 200 cc. of water and having a reaction of P_H 4.0 were transferred to the wash bottles. Series No. 1 was aerated continuously for 5 days. Air from which the oxygen was removed with alkaline pyrogallol[1] was passed through the duplicate series No. 2.

After 5 days the reaction of each sulphur suspension was tested and found to be P_H 1.4 in Series No. 1, and P_H 1.8 in Series No. 2. The hydrogen-ion concentrations were adjusted to P_H 5.0 with M/10 Na_2HPO_4, and toxicity tests were made in closed-ring cells in the usual way. For a comparison a freshly made solution of colloidal sulphur was also tested. Spores of *Sclerotinia cinerea* and *Botrytis Alii* were used as indicators of the toxicity. The results are recorded in table VI. In the table the dilution of sulphur used was 1–20,000 for *Sclerotinia cinerea* and 1–2,000 for *Botrytis Alii*.

[1] One part pyrogallol, 5 parts NaOH, and 30 parts H_2O.

TABLE VI

TOXICITY OF PRE-DRIED AND OF AERATED SULPHUR SUSPENSIONS. THE FIGURES REPRESENT PERCENTAGE OF GERMINATION (AVERAGE OF DUPLICATE CULTURES MADE AT DIFFERENT TIMES)

Organism	Treatment of sulphur				
	Control, without sulphur	Freshly prepared	Air-dried	Aerated $+O_2$	Aerated $-O_2$
Botrytis Alii	71	15	10	2	8
Sclerotinia cinerea	57	8	12	11	7

The results show that drying and continuous aeration for 5 days has no appreciable effect upon the toxicity of this form of colloidal sulphur. Further tests will be required to determine whether there is any influence from more protracted periods of drying and aerating. Likewise, further investigations are necessary to determine what acid or acids are responsible for changing the P_H of the colloidal sulphur solution during the aeration. It was found that the aeration of sulphur "flour" resulted in a change in reaction toward the acid range within a very short time.

THE INFLUENCE OF TEMPERATURE UPON THE TOXICITY OF COLLOIDAL SULPHUR

It has been shown by Doran ('22) that the fungicidal value of sulphur increases with the rise of temperature. Butler ('23) found that the temperature was an important factor in controlling snapdragon rust by the use of sulphur dust. Below 15° C. sulphur was found to be ineffective.

An experiment was arranged to test the influence of a restricted temperature range upon the toxicity of a colloidal sulphur preparation. A suspension containing 1 gm. of colloidal sulphur (SO_2-H_2S form) in 200 cc. of water was freshly prepared. The reaction of this suspension was P_H 4.0. A dilution of 1 to 8,000 was made from this suspension in the slightly buffered solution P_H 4.2. This concentration of sulphur was known not to inhibit germination of spores at ordinary temperature. Toxicity tests were made with spores of *Botrytis cinerea* at the temperatures 18°, 22°, 24°, and 28° C. Controls without sulphur were also

run at the same time. These tests were made in duplicate and conducted at two different times. The results are shown in table VII.

TABLE VII

THE EFFECT OF TEMPERATURE UPON THE TOXICITY OF A COLLOIDAL SULPHUR SUSPENSION. THE FIGURES REPRESENT PERCENTAGE OF GERMINATION

Organism	Form of sulphur	Temperature ° C.			
		19	22	24	28
Botrytis cinerea	Control	67	70	81	40
	Colloidal sulphur	18	22	17	8

Toxicity was exhibited at the lowest temperature tested, though the higher temperatures increased the toxicity to a considerable extent. It would be of importance to test the effect of a wider range of temperatures and with a variety of organisms.

Practical Application

After it was found that colloidal sulphur prepared by the methods herein described was effective in inhibiting the germination of spores of certain economic fungi, as usually tested in the laboratory, it was next necessary to demonstrate the fungicidal value of these materials by practical field applications. Arrangements were made with Dr. L. F. Nickell of the Monsanto Chemical Works, East St. Louis, Ill., for the use of equipment to manufacture a sufficient quantity of materials for field tests.

The materials were prepared according to methods already described, with certain modifications to suit the available equipment. A 500-gallon wooden tub, fitted with a steam coil, agitators, cover, and a flue provided with a steam jet were used for the purpose.

The hypo-H_2SO method was necessarily modified, since the concentrated H_2SO_4 could not be used in the tub because of the iron agitator and steam coil. The method used was as follows: Seven hundred pounds of hypo were dissolved in 75 gallons of water and the solution warmed with the steam coil to 50° C. Ten pounds of glue were dissolved by means of steam and this

was added to the hypo solution. One hundred pounds of commercial H_2SO_4, sp. gr. 1.84, were added slowly by means of a siphon. The mixture was then adjusted to P_H 4.2 with sulfocide, 1 part to 5 parts of water. The agitator was kept in motion during the entire process.

The lime sulphur method was slightly modified as follows: Commercial lime sulphur solution was diluted with 4 parts of water and the mixture warmed to 40° C. Ten pounds of glue, previously dissolved by means of steam, were added for each 100 gallons of the diluted lime sulphur. The reaction of the lime sulphur mixture was then adjusted to P_H 4.2 with HNO_3, 1 part to 20 parts of water. The agitator was kept in motion throughout the process. It was originally planned to allow the mixture to set over night with the agitator in motion in order to free the mixture of H_2S. Due to the short interval of time to which the writer was restricted in the use of the apparatus, another and more rapid method was used to eliminate the H_2S. Therefore, the mixture was heated to 70° C. and aerated several hours by means of an air pump. This aeration and heating unfortunately destroyed the stability of the solution to a considerable extent. Additional quantities of the mixture were prepared in a similar way by using 1 to 20 H_2SO_4.

The materials thus prepared were sent to the Missouri Agricultural Experiment Station, and to Adams and Chester Counties, Pennsylvania, to be used in tests being made for the control of apple diseases. Mr. R. C. Walton, of the Pennsylvania Agricultural Experiment Station, had charge of the field tests in Pennsylvania.

Due to the seasonal conditions very little disease appeared in the apple orchard at the Missouri Station. Therefore, no comparative results of value were obtained.

The results of the tests conducted in the commercial orchards of Pennsylvania were very favorable, particularly in the case of the preparation made from lime sulphur. These materials gave very efficient control of apple scab, a disease which was severe in Pennsylvania during that season. The details of these results will presumably be published separately (Walton, '26). The injury to foliage and fruit was a minimum in the plots sprayed

with these materials. The writer observed these plots in the late summer, and it was then evident that the foliage of the trees sprayed with the mixture prepared from lime sulphur and HNO_3 was of a much darker green color. Furthermore, the growers found the use of these materials satisfactory because of the ease of handling and applying.

Colloidal sulphur was made in the laboratory by the SO_2-H_2S method for field tests. The method used was the same as has already been described except that a 10-gallon glass carboy was used as a reaction tank for the SO_2 and H_2S. Large earthenware jars (especially made for the Chemical Department, Washington University) were used for H_2S generators. The product produced by this method was sent to the Michigan, Missouri, and New York Agricultural Experiment Stations, and to Adams County and Chester County, Pennsylvania, for field tests.

The results of these tests were not so promising as was anticipated, due probably to two factors: (1) The dilution of the sulphur used was probably too great; and (2), as has already been indicated, the sulphur crystallized in a short time after it was prepared. The technique of large-scale preparation, therefore, requires further detailed study.

Conclusions

1. Methods have been devised for preparing promising colloidal sulphur suspensions for use in practical spraying.

2. The stability of colloidal sulphur preparations was found to depend upon the method of mixing the chemicals, the temperature of solutions, and upon the introduction of protective colloids.

3. Of the emulsoid colloids that it has been practicable to employ, glue has proved to be the most effective in preserving the stability of colloidal sulphur suspensions.

4. The use of weak alkalies in adjusting suspensions prepared from hypo and H_2SO_4 was found necessary; and for this purpose Na_2CO_3 and Na_2HPO_4 gave the best results.

5. In preparing colloidal sulphur from lime sulphur, weak, rather than strong, solutions of HNO_3 and H_2SO_4 were more satisfactory.

6. The colloidal sulphur mixtures prepared by the methods

devised in the course of this work were found to be toxic to all forms of fungi tested under laboratory conditions.

7. Greenhouse tests with a variety of plants have shown that the products spread well and that they adhere to the foliage; at the same time they do not cause injury under the conditions tested.

8. Colloidal sulphur freshly prepared from SO_2 and H_2S was effective for the control of carnation rust in the greenhouse.

9. It would seem to be demonstrated that the toxicity of different forms of colloidal sulphur may be ascribed to different toxic substances. The properties of the toxic substances exhibited by colloidal sulphur prepared from SO_2 and H_2S are different from the toxic substance (pentathionic acid) exhibited by Young's hydrophilic colloidal sulphur.

10. There is a gradual increase in toxicity of colloidal sulphur formed from SO_2 and H_2S, beginning at P_H 4.2 and continuing to P_H 5.4, while at higher P_H values there is a rapid increase. Young's hydrophilic colloidal sulphur exhibits the greatest toxicity in the range P_H 4.2 to 5.4.

11. Colloidal sulphur prepared from SO_2 and H_2S in paste form will crystallize in about 1 month. This crystallization destroys the toxic property.

12. Colloidal sulphur preparations have greatest stability between P_H 3.0 and 5.4. The loss of stability is accompanied by a destruction of the toxic property.

13. The toxic constituent of colloidal sulphur, when freshly prepared from SO_2 and H_2S, is slightly volatile and is liberated most rapidly at a higher P_H than 4.2.

14. Desiccation and aeration do not destroy the stability or the toxic component of colloidal sulphur prepared from SO_2 and H_2S.

15. Colloidal sulphur prepared from SO_2 and H_2S is most toxic at higher temperatures.

16. When manufactured for field spraying tests the methods for preparing colloidal sulphur were modified to suit the equipment available. Prepared in this way, certain of the products gave excellent control of apple scab during the summer of 1924.

The writer takes great pleasure in extending thanks for many suggestions and criticisms to Dr. B. M. Duggar, who directed the work; to Dr. H. C. Young, of the Ohio Agricultural Experiment Station for many suggestions; to Dr. George T. Moore, for the privileges and facilities of the Missouri Botanical Garden; and to the Crop Protection Institute for the necessary funds and materials.

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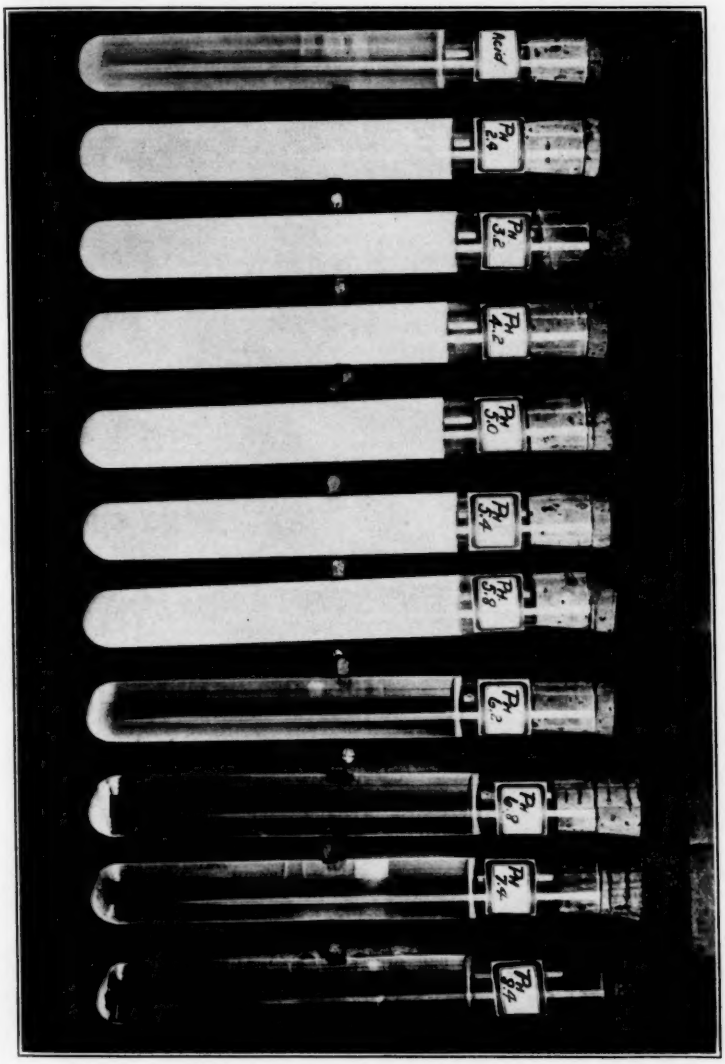
Explanation of Plate

PLATE 20

Effect of the hydrogen-ion concentration (P_H) upon the stability of colloidal sulphur solution.



TISDALE—COLLOIDAL SULPHUR

GENERAL INDEX TO VOLUME XII

New scientific names of plants and the final members of new combinations are printed in **bold face** type; synonyms and page numbers having reference to figures and plates, in *italics;* and previously published scientific names and all other matter, in ordinary type.

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